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REC'D 14 SEP 2000

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 9FPO-04-08	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/KR99/00347	International filing date (day/month/year) 30 JUNE 1999 (30.06.1999)	Priority date (day/month/year) 30 JUNE 1998 (30.06.1998)
International Patent Classification (IPC) or national classification and IPC IPC7 C12N 15/19, C12N 15/79, C12N 5/10, C12N 5/10		
Applicant DAEWOONG PHARMACEUTICAL CO., LTD. et al		

<p>1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of <u>4</u> sheets, including this cover sheet.</p> <p><input type="checkbox"/> This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).</p> <p>These annexes consist of a total of _____ sheets.</p>	
<p>3. This report contains indications relating to the following items:</p> <p>I <input checked="" type="checkbox"/> Basis of the report</p> <p>II <input type="checkbox"/> Priority</p> <p>III <input type="checkbox"/> Non-establishment of opinion with regard to novelty, inventive step and industrial applicability</p> <p>IV <input type="checkbox"/> Lack of unity of invention</p> <p>V <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement</p> <p>VI <input type="checkbox"/> Certain documents cited</p> <p>VII <input checked="" type="checkbox"/> Certain defects in the international application</p> <p>VIII <input type="checkbox"/> Certain observations on the international application</p>	

Date of submission of the demand 14 JANUARY 2000 (14.01.2000)	Date of completion of this report 30 AUGUST 2000 (30.08.2000)
Name and mailing address of the IPEA/KR Korean Industrial Property Office Government Complex-Taejon, Dunsan-dong, So-ku, Taejon Metropolitan City 302-701, Republic of Korea Facsimile No. 82-42-472-7140	Authorized officer AHN, Mi-Chung Telephone No. 82-42-481-5593



INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/KR99/00347

I. Basis of the report

1. With regard to the elements of the international application:*

- ☒ the international application as originally filed
- ☐ the description:
pages _____, as originally filed
pages _____, filed with the demand
pages _____, filed with the letter of _____
- ☐ the claims:
pages _____, as originally filed
pages _____, as amended (together with any statement) under Article 19
pages _____, filed with the demand
pages _____, filed with the letter of _____
- ☐ the drawings:
pages _____, as originally filed
pages _____, filed with the demand
pages _____, filed with the letter of _____
- ☐ the sequence listing part of the description:
pages _____, as originally filed
pages _____, filed with the demand
pages _____, filed with the letter of _____

2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language _____ which is

- ☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide** and/or amino acid sequence disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☐ The amendments have resulted in the cancellation of:

- ☐ the description, pages _____
- ☐ the claims, Nos. _____
- ☐ the drawings, sheet _____

5. ☐ This opinion has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed." and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).

** Any replacement sheet containing such amendments must be referred to under item I and annexed to this report.

INTERNATIONAL PRELIMINARY EXAMINATION

International application No.

PCT/KR99/00347

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

I. Statement

Novelty (N)	Claims	1-12	YES
	Claims		NO
Inventive step (IS)	Claims	2, 4, 7-10	YES
	Claims	1, 3, 5, 6, 11 & 12	NO
Industrial applicability (IA)	Claims	1-12	YES
	Claims		NO

2. Citations and explanations (Rule 70.7)

D1 : WO 96/25498

D2 : Park et al. J. Biological Chemistry. January 1998. 273(1) : 256-261

I. Novelty

The claimed invention relates to human thrombopoietin (hTPO) derivatives which purpose the improved activity of platelet production by employing the strategy of exploitation of N-glycosylation of TPO. Even if the prior arts, D1 and D2, have described the strategy employing modulation of N-glycosylation sites for generating analogs of TPO, specific glycosylation sites manipulated in TPO analogs of the prior arts are quite different from those of the claimed invention (See claim 8 in D1 and Table 1 in D2). Accordingly, the subject matter of claims 1-12 seems to be novel {Art. 33(2)}.

II. Inventive step

The significance of the claimed invention compared to the prior arts, D1 or D2, is to develop hTPO derivatives with the improved activity of platelet production. However, only 4 hTPO derivatives (40433, 40434, 40449 and 40458) among various derivatives presented in the claimed invention have shown higher activity than native hTPO (See p.21 & Fig. 6, 7a or 7b). Since modulation of N-glycosylation sites to develop TPO derivatives should be obvious to a person skilled in the art, it is not possible to acknowledge inventive step for claims related to TPO derivatives which failed to enhance TPO activity. In such circumstance, claims 1, 3, 5, 6, 11 and 12 can not be regarded as satisfying the requirement of inventive step {Art. 33(3)}.

III. Industrial Applicability

Claims 1-12 meet the criteria for industrial applicability set out in Article 33(4).

INTERNATIONAL PRELIMINARY EXAMINATION

International application No.

PCT/KR99/00347

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

Expressions in line 4 on page 10 (hPO) and line 23 on page 24 (0.01 000) are unclear, thus failing to meet the requirement of Rule 5.1.

PATENT COOPERATION TREATY

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NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

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Date of mailing (day/month/year)
07 February 2000 (07.02.00)

International application No.
PCT/KR99/00347

Applicant's or agent's file reference
9FPO-04-08

International filing date (day/month/year)
30 June 1999 (30.06.99)

Priority date (day/month/year)
30 June 1998 (30.06.98)

Applicant

CHUNG, Joo, Young et al

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:
14 January 2000 (14.01.00)

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2. The election ☒ was
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made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

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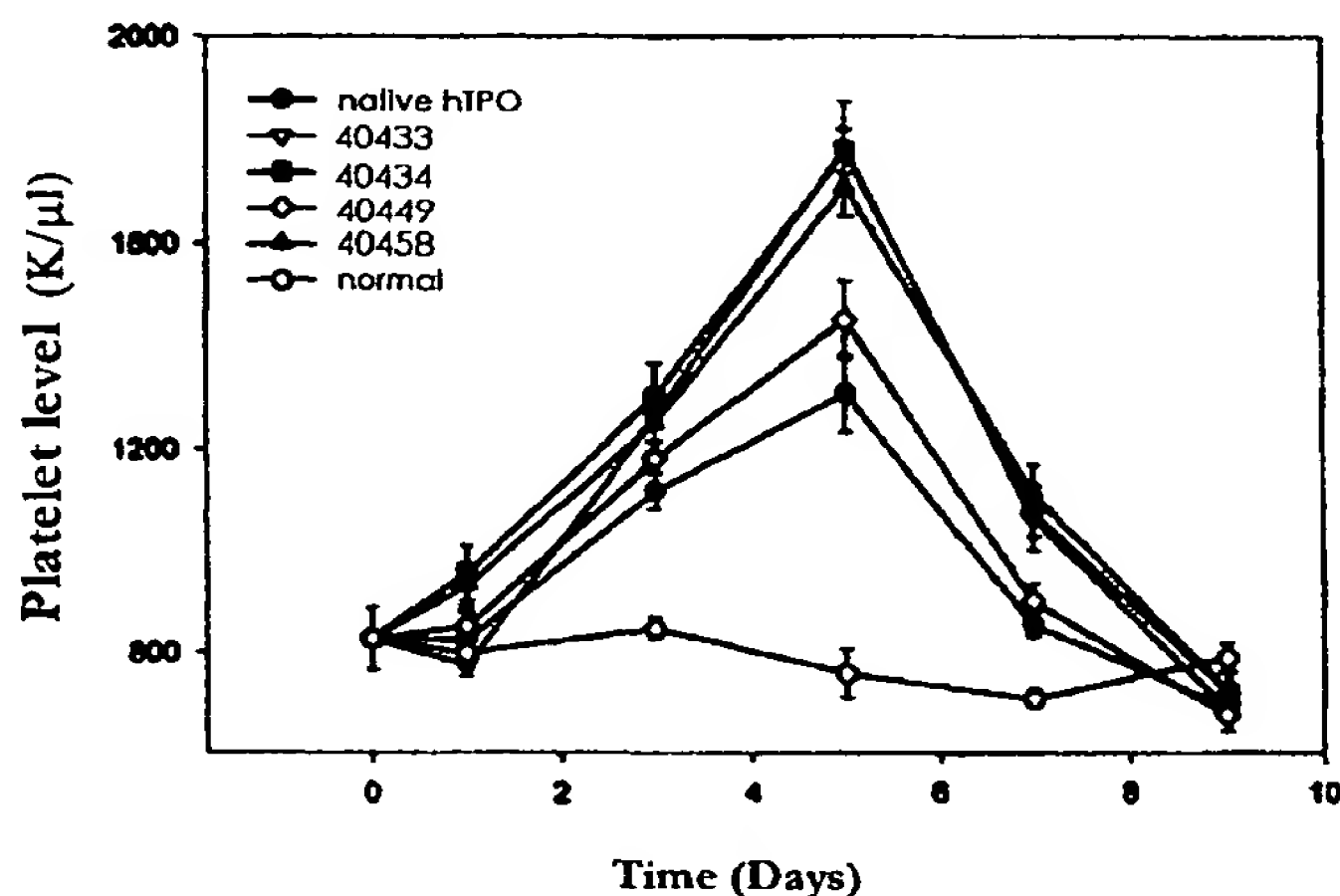
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/19, 15/79, 5/10, A61K 38/19		A1	(11) International Publication Number: WO 00/00612
			(43) International Publication Date: 6 January 2000 (06.01.00)
(21) International Application Number: PCT/KR99/00347 (22) International Filing Date: 30 June 1999 (30.06.99) (30) Priority Data: 199825935 30 June 1998 (30.06.98) KR 199925143 29 June 1999 (29.06.99) KR (71) Applicant (for all designated States except US): DAEWOONG PHARMACEUTICAL CO., LTD. [KR/KR]; 163-3 Samsung-dong, Kangnam-ku, Seoul 135-715 (KR). (72) Inventors; and (75) Inventors/Applicants (for US only): CHUNG, Joo, Young [KR/KR]; #210-1204 Hanshin Apt., Imae-dong, Pundang-ku, Sungnam-si, Kyunggi-do 463-060 (KR). PARK, Sang, Kyu [KR/KR]; 177-3 Mook-1-dong, Chungrang-ku, Seoul 131-141 (KR). JU, Sang, Myoung [KR/KR]; #408-401 Hansol Jookong Apt., Chongja-dong, Pundang-ku, Sungnam-si, Kyunggi-do 463-010 (KR). AHN, Hyea, Kyung [KR/KR]; #609-1305 Imaechon Chongku Apt., Imae-dong, Pundang-ku, Sungnam-si, Kyunggi-do 463-060 (KR). LIM, Seung, Wook [KR/KR]; #106-702 Doosan Apt., 2024 Shinheung-dong, Soojung-ku, Sungnam-si, Kyunggi-do 461-160 (KR). CHANG, Woo, Ik [KR/KR]; #409-1103 Halla Jookong Apt., 1156-1			Sanbon-dong, Koonpo-si, Kyunggi-do 435-040 (KR). PARK, Seung, Kook [KR/KR]; #409 Sanho Apt., San 19-4 Sangdaewon-dong, Joongwon-ku, Sungnam-si, Kyunggi-do 463-120 (KR). KOH, Yeo, Wook [KR/KR]; #126-601 Shibom Hanshin Apt., Seohyun-dong, Pundang-ku, Sungnam-si, Kyunggi-do 463-050 (KR). PARK, Ji, Soo [KR/KR]; 538-59 Donam-dong, Sungbuk-ku, Seoul 136-060 (KR). (74) Agent: LEE, Won-Hee; Suite 805, Sung-ji Heights II, 642-16 Yoksam-dong, Kangnam-ku, Seoul 135-080 (KR). (81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments. With an indication in relation to deposited biological material furnished under Rule 13bis separately from the description.

(54) Title: A NOVEL HUMAN THROMBOPOIETIN MUTEIN



(57) Abstract

The present invention relates to novel human thrombopoietin (hTPO) derivatives, and to process of preparation thereof. Particularly, sugar chains are introduced into native hTPO by substituting amino acids such as asparagine for amino acids at specific positions in native hTPO, preparing novel hTPO derivatives with high activities enhancing the platelet production *in vivo*. Therefore, the novel hTPO derivatives of this invention may be useful for the treatment of thrombocytopenia associated with anticancer therapy or the transplantation of bone marrow.

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A NOVEL HUMAN THROMBOPOIETIN MUTEINFIELD OF THE INVENTION

The present invention relates to novel human
5 thrombopoietin (; hTPO) derivatives with high
activities enhancing the platelet production in vivo,
and to process of preparation thereof.

Particularly, this invention relates to novel hTPO
derivatives wherein sugar chains are introduced by
10 substituting amino acids such as asparagine for amino
acids at specific positions in native hTPO; to
nucleotide sequences encoding the hTPO derivatives; to
expression vectors containing the nucleotide sequences;
to process of construction thereof; to cell lines
15 transformed with the vectors; and to process of
preparing the hTPO derivatives thereby.

BACKGROUND

Thrombocytopenia is the disease of platelet
20 deficiency caused by anticancer therapy, bone marrow
graft and so on. In the process of anticancer therapy
or bone marrow graft, megakaryocyte colony forming
cells, the platelet precursor cells in bone marrow, are
disrupted, and this leads to platelet deficiency.
25 Thrombocytopenia patient is subject to bleeding in

response to a light trauma, and more serious patient becomes bleeding without trauma. Bleeding is often fatal in this case since the blood is not stanchd at all.

5 The current therapy for thrombocytopenia is nothing but the platelet transfusion. However, several problems and side effects are associated with this therapy, such as insufficient donors, transfusion-mediated infection with e.g. HIV (human
10 immunodeficiency virus) and hepatitis viruses, the elicitation of immune response, and so on.

Platelet is a component of blood, originated from megakaryocyte precursor cells, and plays a role in the
15 suppression of bleeding. Thrombopoietin (hereafter, referred to as "TPO"), a glycoprotein synthesized and secreted in liver or kidney, regulates the platelet level in blood. TPO accelerates the proliferation and
20 differentiation of the megakaryocyte precursor cells, which is followed by the platelet production (Lok et al., Nature, 369: 565-568, 1994; De savage et al., Nature, 369: 533-568, 1994).

Since a gene encoding TPO was isolated first from human in 1994 (Lok et al., Nature, 369: 565-568, 1994;
25 De savage et al., Nature, 369: 533-568, 1994; Miyazaki et al., Experimental hematol., 22: 838, 1994; WO 95/18858), clinical approaches for thrombocytopenia

have been based on the function of human TPO (hereinafter, referred to as "hTPO"), that is, the regulation of the platelet level.

5 Three different approaches are proceeded in order to improve the activity of native hTPO.

Glycoprotein hTPO is expressed in cells as an inactive precursor comprising 353 amino acids, and the cleavage of signal peptide (21 amino acids) leads to
10 the secretion of active hTPO protein (332 amino acids) out of the cells. The amino acid sequence of hTPO is divided into two regions. The N-terminal region comprising 151 amino acids contains catalytic site, and shows high similarity to that of erythropoietin (; EPO).
15 The other region, C-terminal region is presumed to have a key role in the extracellular secretion and in vivo stability of hTPO.

The first method for modifying native hTPO relates to the deletion of the C-terminal region or the
20 addition of new amino acids to the deleted hTPO.

In support of this approach, Amgen INC. developed various hTPO derivatives such as hTPO₁₅₁ (consisting of amino acids 1-151), hTPO₁₇₄ (consisting of amino acids 1-174) and the hTPO₁₆₃ supplemented with methionine-
25 lysine in its N-terminal. However, these derivatives proved to show lower hTPO activity in vivo than native hTPO, although their activities were maintained in

vitro (WO 95/26746, WO 95/25498).

In addition, Genentech INC. prepared from *E. coli* a recombinant hTPO₁₅₃ derivative having an N-terminal methionine (WO 95/18858). Kirin produced diverse hTPO
5 derivatives with C-terminal deletion and hTPO₁₆₃ derivatives with substitution, deletion, or insertion at a specific amino acid residue (WO 95/21920). Other hTPO derivatives with C-terminal deletion were provided by Zymogenetics INC. (WO 95/21920; WO 95/17062) and G.
10 D. Searl (WO 96/23888). These derivatives, however, failed to show higher activity of platelet production in vivo than native hTPO.

The second method is associated with the conjugation of polyethyleneglycol (; PEG) with hTPO
15 fragment, which is exemplified by hTPO₁₆₃-PEG of Amgen INC. (WO 95/26746).

The derivatives according to this method, however, have critical handicaps such as poor stability and safety, since they do not contain C-terminal region
20 that is important for the stability of hTPO and since immune response may be elicited by the shift of their folding structures. Moreover, the qualities of products may be uneven because PEG is not so conjugated at a uniform proportion.

25 The third method exploits the glycosylation of hTPO, which may increase the hTPO activity.

Amgen INC. performed a mutagenesis where a

specific nucleotide in cDNA encoding hTPO was substituted to bear amino acid sequence "Asn-X-Ser/Thr" (where X is any amino acid but proline). The mutated gene was used to prepare hTPO derivatives with C-terminal deletion, which comprised 174 amino acids and into which one or more N-linked glycosylation sites are produced (WO 96/25498).

Korea Research Institute of Biology and Biotechnology (KRIBB) produced a hTPO derivative where one sugar chain is incorporated into the intact native hTPO (Park et al., J. Biol. Chem., 273: 256-261, 1998), distinctive from the Amgen's partial hTPO derivatives.

However, all these derivatives did not show significantly higher levels of hTPO activity.

As described above, although various strategies have been employed to develop hTPO derivatives with enhanced biological activity, all failed to obtain the derivatives with higher in vivo hTPO activities than native hTPO.

20

Generally, numerous proteins exist as proteins adorned by oligosaccharide chains in specific position, i.e. glycoproteins. Two types of glycosylation have been found. In O-linked glycosylation, sugar chain is attached to the hydroxyl group of Ser/Thr residue in the glycoprotein. In N-linked glycosylation, sugar chain is attached to the amide group of "Asn-X-Ser/Thr"

25

(X is any amino acid but proline).

The sugar chain in a glycoprotein exert various effects on the physical, chemical and biological properties such as protein stability and secretion, especially on the biological activity in vivo and pharmacokinetic properties (Jenkins et al., Nature Biotechnological., 14: 975-981, 1996; Liu et al., Act. TIBTECH., 10: 114-120, 1992).

These effects are exemplified by human interferon- γ and glucose transport protein, where amino acid substitution at proper glycosylation site gave rise to the striking decrease in the hTPO activity, suggesting that N-linked sugar chain may have significant effects on the activity of the glycoprotein (Sareneva et al., Biochem. J. 303: 831-840, 1994; Asano et al., FEBS, 324: 258-261, 1993).

However, the introduction of additional sugar chains is not always accompanied with an increase in the catalytic activity of the glycoprotein, as described in the precedent art of Amgen INC. and KRIBB (WO 96/25498; Park et al., J. Biol. Chem., 273: 256-261, 1993). Although additional sugar chains were introduced into these hTPO derivatives, the biological activities of the glycoproteins were rather reduced when compared with native hTPO. According to this observation, it is not the number of sugar chains but the specific glycosylation site that is crucial for

elevating its catalytic activity.

We, the inventors of this invention, have prepared various hTPO derivatives and examined their activities. This invention is performed by disclosing that several hTPO derivatives such as derivative wherein Asn is substituted for Arg¹⁰⁴; derivative wherein Asn is substituted for Thr¹⁹⁵; derivative wherein Asn is substituted for Pro¹⁵⁷ and Arg¹⁶⁴; and derivative wherein Asn is substituted for Leu¹⁰⁵, Arg¹¹⁷ and Arg¹²⁴ produce the remarkably higher levels of platelets than native hTPO does, which is not ever observed in the current hTPO derivatives.

SUMMARY OF THE INVENTION

It is an object of this invention to provide novel hTPO derivatives that show the higher activities enhancing the platelet production in vivo than native hTPO does.

20

In accordance with the present invention, the foregoing objects and advantages are readily obtained.

The present invention provides novel hTPO derivatives with higher activity inducing the platelet production in vivo. Additional sugar chains are introduced into said hTPO derivatives through

substituting amino acids such as asparagine for amino acids at specific positions in native hTPO.

This invention also provides genes encoding said hTPO derivatives.

5 In addition, this invention provides process of preparing said hTPO derivatives, comprising the step wherein said genes are inserted into appropriate vector; the step wherein a host cell is transfected with said vector; and the step wherein the transfected
10 cells are cultured in appropriate medium.

Further features of the present invention will appear hereinafter.

BRIEF DESCRIPTION OF THE DRAWINGS

15 FIG. 1 depicts PCR-based mutagenesis wherein the cDNAs encoding hTPO derivatives are produced, where

1: primer described by SEQ ID NO: 1;

2: primer described by SEQ ID NO: 2;

N: N-primer;

20 C: C-primer;

S: signal sequence.

FIG. 2 depicts the process of linking the mutated genes to pBlueBac4 vector.

FIG. 3 depicts the process of constructing animal
25 expression vectors that the mutated cDNAs are subcloned in pCDT vector.

FIG. 4 presents the result of cell proliferation assay where the activity of M-07e cell proliferation is measured in the presence of hTPO derivatives expressed in animal cells.

5 FIG. 5 presents the in vivo activity of native hTPO, which is determined by measuring the number of platelets in mouse blood after treatment with various doses of native hTPO.

10 FIG 6. presents the in vivo activities of various hTPO derivatives, which are determined by measuring the number of platelets in mouse blood after treatment with hTPO derivatives (36 µg/kg) expressed in animal cells.

15 FIG. 7a and 7b present the in vivo activities of various hTPO derivatives, which are determined by measuring the number of platelets in mouse blood after treatment with hTPO derivatives (10 µg/kg) expressed in animal cells.

20 FIG. 8 depicts the process of constructing the dhfr expression vectors that contain a gene encoding native hTPO or hTPO derivatives.

FIG. 9 presents the result of SDS-PAGE and silver staining with the various fractions obtained in the purification of a hTPO derivative, where

25 lane M: size marker;
 lane 1: culture supernatant;
 lane 2: CM-ion exchange affinity column elutes;
 lane 3: phenylsephrose column elutes;

lane 4: hydroxyapatite column elutes;

lane 5: Q cartridge column elutes.

FIG. 10 presents the in vivo activities of native hPO and various hTPO derivatives, which are determined
5 by measuring the number of platelets in mouse blood after treatment with native hTPO or purified hTPO derivatives (10 µg/kg).

FIG. 11 presents the result of SDS-PAGE and western blot analysis with the purified native hTPO and
10 hTPO derivatives, where

lane M: size marker;

lane 1: native hTPO;

lane 2: hTPO derivative 40433;

lane 3: hTPO derivative 40434;

15 lane 4: hTPO derivative 40449;

lane 5: hTPO derivative 40458;

FIG. 12a and 12b present the result of Western blot analysis, in which the thrombin-digestion pattern of native hTPO (FIG. 12a) or its derivative 40433 (FIG.
20 12b) is shown according to the time after digestion, where

lane M: size marker;

lane 1: Before digestion;

lane 2: 30 minutes after digestion;

25 lane 3: 1 hour;

lane 4: 2 hours;

lane 5: 3 hours;

lane 6: 4 hours;

lane 7: 6 hours.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

5 Hereinafter, the present invention is described in detail.

The present invention provides novel hTPO derivatives with enhanced activity inducing the platelet production in vivo. Additional sugar chains
10 are introduced into said hTPO derivatives through substituting amino acids such as asparagine for amino acids at specific positions in native hTPO.

To develop novel hTPO derivatives with enhanced activity inducing the platelet production in vivo, a
15 variety of hTPO derivatives were prepared, into which one or more sugar chains are introduced through substituting one or more amino acids at specific positions in a hTPO protein. In result, N-linked glycosylation site "Asn-X-Ser/Thr" (where X is any
20 amino acid but proline) is created at the specific positions.

In a preferred embodiment, site-specific mutagenesis using overlap PCR (Cheng et al., Proc. Natl. Acad. Soc. USA, 91: 5695, 1994) was employed to produce
25 the genes encoding hTPO derivatives with specific amino acids substituted at specific position (see FIG 1).

First, the following primer pairs containing mutated sequences were synthesized chemically. These oligonucleotide primer pairs contain the nucleotide sequences corresponding to the mutated amino acid residues, and extend to the 5' or 3' neighboring sequence to the mutated region in hTPO cDNA.

Table 1. Primer pairs for site-specific mutagenesis

Derivative	primer	SEQ ID NO:	Nucleotide sequence
40429	29-N	3	5'-GCTGT GGTGT TGCCC TGTGG-3'
	29-C	4	5'-ACAGG GCAAC ACCAC AGCTC-3'
40430	30-N	5	5'-GGGTT CCGTT TAAAC TCTGC AG-3'
	30-C	6	5'-CTGCA GAGTT TAAAC GGAAC CCAG-3'
40431	31-N	7	5'-AGAGG GTGGA ATTCC CTACA AGCA-3'
	31-C	8	5'-TGCTT GTAGG GAATT CCACC CTCT-3'
40432	32-N	9	5'-GGGCC CCGTT GACGC AGA-3'
	32-C	10	5'-TCTGC GTCAA CCGGG CCC-3'
40433	33-N	11	5'-GGACT AGAGA CGTGT TGCTG GGGAC-3'
	33-C	12	5'-GTCCC CAGCA ACACG TCTCT AGTCC-3'
40434	34-N	13	5'-GAAGC CCAGA TCCGT TAGTT CTGGC-3'
	34-C	14	5'-GCCAG AACTA ACGGA TCTGG GCTTC-3'
40458	58-N	15	5'-AGCTG TGGTG TTTGG GGCCC GC-3'
	58-C	16	5'-GCGGG CCCC AACCAC CACAG CT-3'
	33-N	11	5'-GGACT AGAGA CGTGT TGCTG GGGAC-3'
	33-C	12	5'-GTCCC CAGCA ACACG TCTCT AGTCC-3'
40459	59-N	17	5'-CTAGA GAGGT GCTGT TGACA GCTGT G-3'
	59-C	18	5'-CACAG CTGTC AACAG CAGCA CCTCT CTAG-3'
40460	60-N	19	5'-GGTGG GTGGG GTCCG GTTGA CGCAG AGG-3'
	60-C	20	5'-CCTCT GCGTC AACCG GACCC CACCC ACC-3'
	33-N	11	5'-GGACT AGAGA CGTGT TGCTG GGGAC-3'
	33-C	12	5'-GTCCC CAGCA ACACG TCTCT AGTCC-3'
40461	61-N	21	5'-TCTGC TGGGG GAAGC GTTGG TGGGT GG-3'
	61-C	22	5'-CCACC CACCA ACGCT TCCCC CAGCA GA-3'
	33-N	11	5'-GGACT AGAGA CGTGT TGCTG GGGAC-3'
	33-C	12	5'-GTCCC CAGCA ACACG TCTCT AGTCC-3'
40462	62-N	23	5'-CAGTG TGAGG GTTAG ATTGG TTCTG CTG-3'
	62-C	24	5'-CAGCA GAACC AATCT AACCC TCACA CTG-3'
40463	63-N	25	5'-CAGTG TGAGG TTTAG AGAGG TT-3'
	63-C	26	5'-AACCT CTCTA AACCT CACAC TG-3'
	33-N	11	5'-GGACT AGAGA CGTGT TGCTG GGGAC-3'
	33-C	12	5'-GTCCC CAGCA ACACG TCTCT AGTCC-3'

Overlap PCR was performed wherein the established vector pBlue404 (KOREA APPLICATION NO. 97-7512) containing hTPO cDNA was employed as a template. On the one hand, the oligonucleotide (SEQ ID NO: 1) encoding hTPO signal peptide and one of oligonucleotides (N-primer series in Table 1) encoding mutated sequences were employed as PCR primers. On the other hand, the oligonucleotide (SEQ ID NO: 2) containing hTPO C-terminal ORF and stop codon and one of oligonucleotides (C-primer series in Table 1) encoding mutated sequences were employed as PCR primers.

The overlap PCR products contain the DNA sequences covering from N-terminal signal sequence to mutated region and the DNA sequences covering from mutated region to C-terminal region, respectively.

To obtain the full-length hTPO cDNA sequence containing the target site for amino acid substitution, PCR was done where the two overlap PCR products were employed as a template and two oligonucleotides (SEQ ID NO: 1 and NO: 2) were employed as PCR primers.

Through aforesaid processes, 1078-bp full-length cDNA sequences encoding hTPO derivatives were prepared, which contained a variety of mutated sequences (see FIG. 1).

In a further embodiment, vectors containing the

cDNAs for hTPO derivatives were constructed in order to obtain the expression vectors containing the cDNAs and finally to produce the cell lines transfected with the expression vectors.

5 Particularly, the established vector pBlueBac4 and each cDNA encoding hTPO derivative were digested with *Bgl*III and *Eco*RI restriction enzymes, respectively. Then the two DNA fragments were linked with T4 DNA ligase to construct vectors containing the hTPO
10 derivative cDNA (see FIG. 2).

 The resulting vectors are illustrated by Table 2, which gives the names of the vectors, the mutated sequences encoding hTPO derivatives, and the amino acid residues modified in accordance with the mutation.

15 The amino acid sequences of hTPO derivatives of this invention are represented by a method where they are described with the amino acid residue substituted and a specified position in the amino acid sequence of native hTPO (SEQ ID NO: 30). For instance, a hTPO
20 derivative of this invention, 40430, may be also referred to as "[Asn¹⁰⁸] hTPO" corresponding to the amino acid sequence described by SEQ ID NO: 30, except for asparagine substituted for the amino acid residue 108.

25

Table 2. The substituted amino acid and nucleotide sequences in the vectors containing hTPO derivative

cDNAs

Vectors	substituted amino acid	Mutated nucleotide
pBlue29	R ¹⁵⁷ → N ¹⁵⁷	AGG → AAC
pBlue30	L ¹⁶² → N ¹⁶²	CTT → AAT
pBlue31	G ¹⁴⁶ G ¹⁴⁷ → G ¹⁴⁶ N ¹⁴⁷	GGAGGG → GGGAAT
pBlue32	R ¹⁵³ → N ¹⁵³	AGG → AAC
pBlue33	R ¹⁶⁴ T ¹⁶⁵ → N ¹⁶⁴ T ¹⁶⁵	AGAACC → AACACG
pBlue34	T ¹⁵³ G ¹⁵⁴ → N ¹⁵³ G ¹⁵⁴	ACTGGT → AACGGA
pBlue58	P ¹⁵⁷ → N ¹⁵⁷	CCC → AAC
	R ¹⁶⁴ T ¹⁶⁵ → N ¹⁶⁴ T ¹⁶⁵	AGAACC → AACACG
pBlue59	R ¹⁶² , R ¹⁶⁴ → N ¹⁶² , S ¹⁶⁴	CCC, AGA → AAC, AGC
pBlue60	R ¹⁵³ , A ¹⁵⁵ → N ¹⁵³ , T ¹⁵⁵	AGG, GCC → AAC, ACC
	R ¹⁶⁴ T ¹⁶⁵ → N ¹⁶⁴ T ¹⁶⁵	AGAACC → AACACG
pBlue61	T ¹⁵⁹ , V ¹⁶¹ → N ¹⁵⁹ , S ¹⁶¹	ACA, GTC → AAC, TCC
	R ¹⁶⁴ T ¹⁶⁵ → N ¹⁶⁴ T ¹⁶⁵	AGAACC → AACACG
pBlue62	S ¹⁶⁶ , V ¹⁶⁸ → N ¹⁶⁶ , T ¹⁶⁸	TCT, GTC → AAT, ACC
pBlue63	R ¹⁶⁴ T ¹⁶⁵ → N ¹⁶⁴ T ¹⁶⁵	AGAACC → AACACG
	V ¹⁶⁸ → N ¹⁶⁸	GTC → AAC

In another preferred embodiment, the expression
 5 vectors, which contain the hTPO derivative cDNA
 sequences, were constructed in order to be introduced
 into an animal cells.

Specifically, pCDT vector was prepared through the
 insertion of native hTPO cDNA into the established
 10 vector pCDNA3.1. The pCDT and the vectors containing
 hTPO derivative genes, such as pBlue29, pBlue30,
 pBlue31, pBlue32, pBlue33, pBlue34, pBlue58, pBlue59,
 pBlue60, pBlue61, pBlue62 and pBlue63 were digested
 with *NheI* and *EcoRI* enzymes. Then, these fragments
 15 were ligated with T4 DNA ligase to obtain animal
 expression vector containing each hTPO derivative gene

(see FIG. 3 and Table 3).

Table 3. The substituted amino acid and nucleotide sequences in animal expression vectors containing hTPO derivative cDNAs.

Expression vector	Mutated amino acid	Mutated base
p40429	R ¹¹⁷ → N ¹¹⁷	AGG → AAC
p40430	L ¹⁰⁸ → N ¹⁰⁸	CTT → AAT
p40431	G ¹⁴⁶ G ¹⁴⁷ → G ¹⁴⁶ N ¹⁴⁷	GGAGGG → GGG AAT
p40432	R ¹⁵³ → N ¹⁵³	AGG → AAC
p40433	R ¹⁶⁴ T ¹⁶⁵ → N ¹⁶⁴ T ¹⁶⁵	AGAACC → AACACG
p40434	T ¹⁹³ G ¹⁹⁴ → N ¹⁹³ G ¹⁹⁴	ACTGGT → AACGGA
p40435	p40429 + p40431	
p40436	p40429 + p40433	
p40437	p40430 + p40431	
p40438	p40430 + p40433	
p40439	p40431 + p40433	
p40446	p40429 + p40431 + p40433	
p40447	p40430 + p40431 + p40433	
p40449	p40429 + p40430 + p40433	
p40458	P ¹⁵⁷ → N ¹⁵⁷ R ¹⁶⁴ T ¹⁶⁵ → N ¹⁶⁴ T ¹⁶⁵	CCC → AAC AGAACC → AACACG
p40459	R ¹⁶² , R ¹⁶⁴ → N ¹⁶² , S ¹⁶⁴	CCC, AGA → AAC, AGC
p40460	R ¹⁵³ , A ¹⁵⁵ → N ¹⁵³ , T ¹⁵⁵ R ¹⁶⁴ T ¹⁶⁵ → N ¹⁶⁴ T ¹⁶⁵	AGG, GCC → AAC, ACC AGAACC → AACACG
p40461	T ¹⁵⁹ , V ¹⁶¹ → N ¹⁵⁹ , S ¹⁶¹ R ¹⁶⁴ T ¹⁶⁵ → N ¹⁶⁴ T ¹⁶⁵	ACA, GTC → AAC, TCC AGAACC → AACACG
p40462	S ¹⁶⁶ , V ¹⁶⁸ → N ¹⁶⁶ , T ¹⁶⁸	TCT, GTC → AAT, ACC
p40463	R ¹⁶⁴ T ¹⁶⁵ → N ¹⁶⁴ T ¹⁶⁵ V ¹⁶⁸ → N ¹⁶⁸	AGAACC → AACACG GTC → AAC

The scope of this invention includes not only DNA sequences of Table 3 but also other DNA sequences corresponding to the amino acid sequences of Table 3, based on the degeneracy of genetic code. In other

words, all of DNA sequences encoding hTPO derivatives that contain the modified amino acids of Table 3 may be employed as a mutant hTPO gene.

For example, a hTPO derivative, which may be prepared from an expression vector p40433, includes a polypeptide [Asn¹⁶⁴] hTPO, which may be encoded not only by DNA sequence of SEQ ID NO: 31 but also by degenerate DNA sequences.

To confirm the insertion of mutated sequences into the vector, the DNA sequencing of PCR products may be employed. Alternatively, if the overlap PCR primers are designed to contain a new restriction site or to delete a wild-type restriction site, the restriction map of the vector may be used to examine mutagenesis. If an expression vector p40433, for example, has a mutated sequence ACACGT in place of wild-type sequence GAACCT, *AflIII* restriction site will be created in p40433. Thus, the digestion of p40433 with *AflIII* can be used to confirm the introduction of mutated sequence.

In a preferred embodiment, hTPO derivatives with two or more amino acid modifications were produced using said expression vectors in order to attach additional sugar chains to the modified amino acid of native hTPO.

Particularly, two kinds of said expression vectors

were digested with appropriate restriction enzymes, and then the resulting fragments were subcloned in said pCDT vector to construct expression vectors which contain the hTPO derivative genes with two or three regions modified. For example, an expression vector p40429 was digested with *NheI* and *BspMI* enzymes to obtain a DNA fragment involved in the amino acid substitution Arg¹¹⁷ → Asn¹¹⁷. In addition, an expression vector p40431 was digested with *BspMI* and *Bsu36I* enzymes to obtain a DNA fragment involved in the amino acid substitution Gly¹⁴⁷ → Asn¹⁴⁷. The resulting two DNA fragments were inserted into the *BspMI*-*Bsu36I* site of the pCDT vector, constructing an expression vector p40435 that contained a DNA sequence encoding hTPO with two amino acid substitutions, Arg¹¹⁷ → Asn¹¹⁷ and Gly¹⁴⁷ → Asn¹⁴⁷. In accordance with this procedure, expression vectors such as p40436, p40437, p40438, p40439, p40446, p40447, p40448, and p40449 were constructed (see Table 3).

20

In a further preferred embodiment, animal cell transformants expressing each hTPO derivative was prepared.

Particularly, said expression vectors were transfected to animal cell line CHO/K-1 through the lipofectamin method, preparing animal cell line expressing each hTPO derivative.

25

According to the name of the expression vector introduced, the transfected lines were designated CHO K-1/p40429, CHO K-1/p40430, CHO K-1/p40431, CHO K-1/p40432 etc., and CHO K-1/p40433 was deposited in
5 Korean Collection for Type Cultures (; KCTC) on June 17, 1998 (Accession NO: KCTC 0495BP).

In another preferred embodiment, hTPO derivatives were prepared, by culturing animal cell lines
10 transfected with the expression vector of this invention.

Particularly, the transfected lines were subcultured in a serum-containing medium on large scale, and then transferred to a secretion medium. Cultured
15 medium was concentrated and dialyzed to obtain hTPO derivatives.

A hTPO derivative isolated from CHO K-1/p40433 is polypeptide [Asn¹⁶⁴] hTPO where asparagine is substituted for Arginine¹⁶⁴ in native hTPO sequence.

20 A hTPO derivative isolated from CHO K-1/p40434 is polypeptide [Asn¹⁹³] hTPO where Asn is substituted for threonine¹⁹³ in native hTPO sequence.

A hTPO derivative isolated from CHO K-1/p40449 is polypeptide [Asn¹⁰⁸, Asn¹¹⁷, Asn¹⁴⁴] hTPO where asparagine
25 is substituted for leucine¹⁰⁸, arginine¹¹⁷ and arginine¹⁴⁴ in native hTPO sequence.

A hTPO derivative isolated from CHO K-1/p40458 is

polypeptide [Asn¹⁵⁷, Asn¹⁶⁴] hTPO where asparagine is substituted for proline¹⁵⁷ and arginine¹⁶⁴ in native hTPO sequence.

5 In accordance with the names of expression vectors, the hTPO derivatives expressed in the animal cells were designated 40429 to 40439, 40446, 40447, 40449, and 40458 to 40463, respectively. Their in vitro activities were estimated by measuring proliferation of
10 megakaryocyte leukemia cell line.

 In result, derivatives such as 40429, 40430, 40432, 40433, 40434, 40437, 40438, 40439, and the like showed higher levels of biological activity than native hTPO did. No significant relationship between the numbers
15 of additional sugar chains and the in vitro activities was observed, since activities were increased or decreased regardless of the number of sugar chains introduced (see FIG. 4).

20 In a preferred embodiment, hTPO derivatives were administered to mouse and then platelet levels were measured in order to investigate the in vivo biological activities of the hTPO derivatives.

 In detail, 8-week-old mice were divided into 4
25 groups according to their weight and then a predetermined concentration of hTPO was subcutaneously administered to mice. After administration, blood was

collected from peripheral vessels of the mice, and platelet levels in blood were measured. While most of derivatives were found to show lower platelet levels than native hTPO did, derivatives 40433, 40434, 40449
5 and 40458 produced platelets at similar or higher efficiencies (see FIG. 6, 7a, or 7b).

These results suggested that hTPO activity in vivo is dependent not on the number of introduced sugar chains but on the specific position of sugar chains.
10 That is, in order to increase the in vivo activity of hTPO, sugar chains should be introduced into specific positions in hTPO, such as amino acid 164, amino acid 193, and so on.

Most notably, platelet levels in 40433-treated
15 group were higher than in native hTPO-treated group, for 2 days from day 3 or 4 after administration, demonstrating that 40433 can be used as a therapeutic agent of thrombocytopenia. The maximum platelet levels in 40433-treated mice were observed on day 5 after
20 administration, reaching 134% of native hTPO activity on day 5, and more than 180% in total.

In another aspect of this invention, in vivo hTPO activities were investigated in purified hTPO
25 derivatives that had produced same or higher platelet levels than native hTPO. To do this, dhfr expression vectors containing the hTPO derivative genes were

constructed, and the resulting vectors were used to prepare cell lines expressing hTPO genes efficiently.

Particularly, *Bam*HI linker was connected to the *Pvu*II-*Sph*I fragment of pSV2-dhfr vector containing dhfr gene. This 1710-bp DNA fragment containing dhfr gene was inserted into pCDT to prepare dhfr expression vector pDCT containing native hTPO gene. Then, the hTPO derivative genes were inserted into pDCT in place of native hTPO gene, constructing dhfr expression vectors pD40433, pD40434, pD40449, and pD40458 (see FIG. 8).

The dhfr expression vectors containing hTPO derivative genes can be readily amplified in the genome of the transfected eukaryotic cells by subculturing the cells. In a preferred embodiment, these vectors were transfected into animal cell line CHO/dhfr(-). The novel transfected cell lines were designated CHO dhfr-/pD40433, CHO dhfr-/pD40434, CHO dhfr-/pD40449, and CHO dhfr-/pD40458, respectively. CHO dhfr-/pD40434, CHO dhfr-/pD40449, and CHO dhfr-/pD40458 were deposited in Korean Collection for Type Cultures (; KCTC) on June 8, 1999 (Accession NO: KCTC 0630BP, KCTC 0631BP, KCTC 0632BP, respectively). Other dhfr vectors containing hTPO derivative genes and its transfected cell lines may be obtained according to the said procedure.

The transfected cell lines can be cultured on large scale, and hTPO derivatives can be purified in

accordance with the established methods. Various column chromatography procedures may be employed to purify hTPO derivatives from cell lines that are transfected with dhfr expression vectors containing
5 said hTPO derivative genes. In a preferred embodiment, CM ion-exchange affinity column, phenyl sepharose column, hydroxylapatite column, and so on were employed (see FIG. 9).

To evaluate the in vivo biological activities of
10 the purified hTPO derivatives, platelet levels were measured according to said process, after the derivatives were administered to mice. In 40433-, 40434-, 40449- and 40458-treated groups, the platelet yields reached 177%, 191%, 126% and 179% of native
15 hTPO-treated group, respectively, for 10 days since the administration (see FIG. 10).

To confirm the introduction of additional sugar chains into hTPO derivatives, SDS-PAGE and subsequent Western blot analysis were performed with the purified
20 native hTPO and hTPO derivatives. In result, the molecular weights of derivatives 40433 and 40434 were larger than that of native hTPO. The molecular weights of 40458 with two additional sugar chains and 40449 with three ones were proportionally increased,
25 depending on the number of sugar chains (see FIG. 11).

To examine the stability of hTPO derivatives, native hTPO and a derivative 40433 were treated with

thrombin, and then the protein bands in Western blot were observed in accordance with the digestion time. In result, 40433 was more stable against digestion with thrombin than native hTPO (see FIG. 12). Thus, it was suggested that increased stability due to glycosylation might contribute to the elevation of in vivo hTPO activity.

The pharmaceutical composition containing the hTPO derivatives of this invention may be prepared in a conventional process, and may be formulated alone or in combination with pharmaceutically acceptable carriers, forming agents, diluents and so on. The composition may be used in the formulation of powders, granules, tablets, capsules, injections, and the like.

Particularly, it may be employed in combination with water, phosphate buffer, extroso solution, albumin solution, antioxidants, dextrin and the like. Preferably, it may be administered intravenously or subcutaneously.

The hTPO derivatives may be administered in still less dose than native hTPO, for example, in a dosage range of about 0.01 000 $\mu\text{g/kg/day}$.

The hTPO derivatives of this invention may be used for the treatment of thrombocytopenia caused by various conditions.

For instance, it may be useful for the treatment

of thrombocytopenia caused by administration of anticancer agents, radiotherapy, bone marrow graft, hepatitis, liver cirrhosis etc. To treat these diseases, the hTPO derivatives may be administered in combination with anticancer agents such as Adriamycin and Cisplatin, and hematopoietic cytokines such as IL-3, MCSF, SCF and EPO.

EXAMPLES

10 Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

Example 1: The PCR-amplification of cDNAs encoding hTPO derivatives

20 To induce site-specific mutagenesis in the gene encoding native hTPO, 12 pairs of oligonucleotides shown in Table 1 were prepared, which contained the specific nucleotide sequences corresponding to the mutated amino acid residues.

The established vector pBlue404 (KOREA PATENT APPLICATION NO. 97-7512) containing hTPO cDNA was employed as a template on which hTPO gene would be amplified.

5 In detail, PCR was carried out, employing 50 ng of pBlue404 as a template. As primers, oligonucleotide (SEQ ID NO: 1) containing the hTPO signal sequence and one of antisense oligonucleotides containing the mutated sequences (N-primers in Table 1) were used.
10 The PCR reactions were performed in 100 μ l total volume containing 4 μ l of the primer solution (40 pmol/ μ l) and 1 μ l of Pfu (; *Pyrococcus furiosus*) polymerase (2.5 u/ μ l; Stratagene, Cat. No. 600153). Thermocycle in the PCR was as follows: 90 sec at 94°C for pre-denaturation;
15 35 amplification cycles comprising 40 sec at 94°C for denaturation, 60 sec at 55°C for annealing and 120 sec at 72°C for elongation; and 5 min at 72°C for post-elongation.

Another PCR was performed in accordance with above
20 reaction. As PCR primers, oligonucleotide (SEQ ID NO: 2) containing hTPO C-terminal ORF and stop codon, and one of sense oligonucleotides containing the mutated sequences (C-primers in Table 1) were employed.

Obtained in the PCR were DNA fragments covering
25 from N-terminal hTPO signal sequence to mutated sequence, and DNA fragments from the mutated sequence to hTPO C-terminal.

The PCR products were brought to 1% agarose gel electrophoresis, and then the DNA bands of interest were cut with a razor and eluted in 50 µl of tertiary distilled water with QIAEX II kit (Qiagen, Cat No. 20021).

To obtain full-length hTPO cDNAs encoding mutated hTPO, PCR in 100 µl final volume was performed, where two series of PCR products (10 ng, respectively) were employed as templates and two oligonucleotides (SEQ ID NO: 1 and NO: 2) as primers. Thermocycle in the PCR was as follows: 90 sec at 94°C for pre-denaturation; 35 amplification cycles comprising 40 sec at 94°C for denaturation, 60 sec at 58°C for annealing and 120 sec at 72°C for elongation; and 5 min at 72°C for post-elongation.

The PCR products were brought to 1% agarose gel electrophoresis, and then the 1078-bp DNA bands were eluted in 30 µl of tertiary distilled water in accordance with said procedure.

To prepare hTPO genes containing two or more regions of mutated DNA sequences, four pairs of primers (the primers 58-N and 58-C, 60-N and 60-C, 61-N and 61-C, 63-N and 63-C) were used in PCR. The full-length cDNAs containing mutated sequence were prepared in accordance with said procedure, and then again brought to site-specific mutagenesis procedure where a primer pair 33-N and 33-C was used.

The modified amino acid and nucleotide sequences in the resulting cDNAs were shown in Table 2.

Example 2: The construction of mammalian expression
5 vectors containing hTPO derivative cDNAs and their
expression in CHO cells

(2-1) Construction of transfer vectors

The genes encoding hTPO derivatives, which was prepared in Example 1, were subcloned in a commercially
10 available vector pBlueBac4 (Invitrogen, Cat. No. V1995-20), as follows.

The PCR products corresponding to each hTPO derivative were digested with *Bgl*III and *Eco*RI enzymes at 37°C for 3 hours, and then 1068-bp DNA fragment was
15 isolated from the reaction mixture by 1% agarose gel electrophoresis. The 4771-bp DNA fragment was also obtained from pBlueBac4 vector digested with *Bgl*III and *Eco*RI enzymes.

To subclone cDNAs encoding hTPO derivatives in the
20 pBlueBac4 vector, two DNA fragments in a molar ratio of cDNA to vector DNA fragment 4:1 were ligated by incubating them with T4 DNA ligase (NEB, Cat. No. 202S) at 16°C for 16 hours. Then, the ligation mixtures were used to transform *E. coli* TOP10F' strain (Invitrogen,
25 Cat. No. C3030-03) with the resulting transfer vectors.

Electroporation method established already was employed to obtain the *E. coli* transformants. After these transformants were cultured in 50 ml of LB medium (10g Trypton, 5g Yeast extract, 10g NaCl in one liter of water) at 37°C for 18 hours, the transfer vectors were obtained from the cultures with Wizard Midiprep kit (Promega, Cat. No. A7640).

These transfer vectors containing hTPO derivative genes were designated pBlue29, pBlue30, pBlue31, pBlue32, pBlue33, pBlue34, pBlue58, pBlue59, pBlue60, pBlue61, pBlue62, and pBlue63, respectively (see FIG.2).

(2-2) Construction of animal expression vectors

To construct recombinant animal expression vectors containing hTPO derivative genes, pCDT was employed which was prepared by inserting wild-type hTPO gene into a commercially available vector pCDNA3.1 (Invitrogen, Cat. No. 790-20).

Particularly, 5 µg of pCDT vector was digested with *EcoRI* and *NheI* enzymes at 37°C for 3 hours, and then 4958-bp DNA fragment was isolated from the reaction mixture by running on 1% agarose gel. The transfer vectors of Example 2-1 were digested with *EcoRI* and *NheI* enzymes, and then 1087-bp DNA fragment was also isolated from each restriction mixture.

To subclone cDNA fragments encoding various hTPO derivatives in the pCDT vector, two DNA fragments were

mixed to 3:1 molar ratio and incubated with T4 DNA
ligase (NEB, Cat No. 202S) at 16°C for 18 hours. Then,
the ligation mixtures were employed to transform *E.*
coli TOP10F' strain (Invitrogen, Cat. No. C3030-03)
5 with the resulting expression vectors. Electroporation
method established already was employed to obtain the *E.*
coli transformants (see FIG. 3). After these
transformants were cultured in 50 ml of LB medium at
37°C for 18 hours, the expression vectors were obtained
10 from the cultures with Wizard Midiprep kit (Promega,
Cat No. A7640). The animal expression vectors
containing hTPO derivative genes were designated p40429,
p40430, p40431, p40432, p40433, p40434, p40458, p40459,
p40460, p40461, p40462, and p40463, respectively (see
15 FIG. 3). The isolated plasmid DNA was digested with
*Nhe*I, *Eco*RI, *Bam*HI and *Bsu*36I enzymes to verify the
insertion of the cDNAs. The mutation in the expression
vectors was confirmed through restriction mapping and
sequencing. The expression vectors were quantified by
20 DNA electrophoresis according to Sambrook et al.
(Sambrook et al., Molecular cloning - A laboratory
manual, 2nd Ed., Cold spring harbor laboratory press,
1987) and used to transfect CHO/K-1 cell line.

25 (2-3) Expression of hTPO derivative genes in CHO cells

The transfection procedure was carried out
according to lipofectamin (Gibco-BRL, Cat. No.

18324012) method. On the day before transfection, CHO/K-1 cells (ATCC CCL-61) were loaded on 6-well microtiter plates at the density of 2×10^5 cells/well. After 24 hours, the cells were once washed with CHO-S-SFM II medium (Gibco-BRL, Cat. No. 12052-098) and 0.8 ml of fresh medium was added to the cells. Meanwhile, 12 μ g of each expression vector was added to 600 μ l of CHO-S-SFM II medium and then mixed with 600 μ l of CHO-S-SFM II medium containing 36 μ l of lipofectamin. After the mixture was incubated at room temperature for 30 min, 200- μ l aliquots of the mixture per one well were added into the cells in 6-well plates. Then the cells were incubated at 37°C for 5 hours in an atmosphere of 5% CO₂. After the addition of 1 ml of medium containing 10% FBS (Gibco-BRL, Cat. No. 16000-036) to the cells, they were further cultured at 37°C for 24 hours in an atmosphere of 5% CO₂. The medium in the plates was replaced with Ham F-12 (Gibco-BRL, Cat. No. 11059) containing 10% FBS, and then the cells were further cultured at 37°C for 72 hours in an atmosphere of 5% CO₂ to prepare a culture for transient expression.

In addition, after the cells in Ham F-12 medium were cultured for 48 hours, cells in one well of 6-well plates were transferred to medium containing 500 μ g/ml of zeocin (Gibco-BRL, Cat. No. R25001) in 100-mm dishes. After the cells were cultured for 7~10 days, zeocin-resistant colonies were identified through microscope.

Cloning cylinder (Bellco, Cat. No. 2090-01010) was used to isolate more than 12 colonies per one hTPO derivative. Gene expression levels were determined by ELISA kit for hTPO (R&D, Cat. No. DTP00), and thereby
5 the cell lines showing the highest expression levels were selected.

Example 3: The construction of mammalian expression
vectors containing hTPO derivative cDNAs with two or
10 more modified regions, and their expression in CHO
cells

To produce hTPO derivatives where two or more modified amino acid regions, mammalian expression vectors of Example 2 were exploited.

15 In order to construct p40435, the expression vector p40429 was digested with *NheI* and *BspMI* enzymes to isolate 494-bp DNA fragment encoding a substituted amino acid (Arg¹¹⁷ to Asn¹¹⁷). Another expression vector p40431 was cut with *BspMI* and *Bsu36I* enzymes to isolate
20 355-bp DNA fragment encoding a substituted amino acid (Gly¹⁴⁷ to Asn¹⁴⁷). Additionally, animal expression vector pCDT containing hTPO cDNA was digested with *NheI* and *Bsu36I* enzymes. The fragments of p40429 and p40431 were inserted into the fragment of pCDT to construct

animal expression vector p40435, which contains cDNA encoding the hTPO derivative with two modified regions (Arg¹¹⁷ to Asn¹¹⁷ and Gly¹⁴⁷ to Asn¹⁴⁷).

5 Another expression vector p40436 is associated with two amino acid substitutions (Arg¹¹⁷ to Asn¹¹⁷ and Arg¹⁶⁴ to Asn¹⁶⁴) and was prepared by inserting the 494-bp fragment of p40429 and 593-bp *Bsp*MI-*Eco*RI fragment of p40433 into pCDT.

10 Expression vectors such as p40437, p40438, and p40439, were prepared in accordance with the above procedure, where two DNA fragments encoding substituted amino acids were isolated from the corresponding vector and inserted into the expression vector pCDT (see Table 3).

15 Other expression vectors such as p40446, p40447, or p40449, were prepared according to a procedure where three DNA fragments encoding substituted amino acids were isolated from the corresponding vector and inserted into pCDT (see Table 3).

20 These eight vectors obtained here were transfected into CHO/K-1 cells in 6-well plates. According to the procedure of Example 2, cultures for transient expression were prepared, and zeocin-resistant colonies were isolated, respectively.

25

Example 4: Estimation of in vitro activities of hTPO derivatives: M-07e cell proliferation assay

To prepare hTPO derivatives, the transfected cell lines of Example 2 and 3 were cultured in Cell Factory (Nunc, Cat. No. 170009) on 10-liter scale. Each transfected cells (5×10^4 cells/ml) were transferred into Cell Factory containing Ham F-12 medium supplemented with 10% FBS. Cultured for 72 hours, the cells were washed once with PBS and then cultured in ExCell medium (JRH, Cat. No. 14311-10L). After the cells were further cultured at 37°C for 96 hours in an atmosphere of 5% CO₂, supernatants were obtained from the culture. The supernatants were concentrated first with pelicon membrane (Millipore, Cat. No. 42PEL60) and second with minitan membrane (Millipore, Cat. No. 80EL004). After concentration, each sample was brought to dialysis in 1 x TNT buffer (10 mM Tris, 0.15 M NaCl, 0.01% Tween 20, pH 7.4) at 4°C for 30 hours, followed by third concentration with Ultrafree (Millipore, Cat. No. UFV2BGC10). The samples were quantified with ELISA kit three times.

Megakaryocyte leukemia cell line M-07e was maintained in RPMI1640 medium (Gibco-BRL, Cat. No. 22400-089) supplemented with GM-CSF (100 u/ml) and 10% FBS.

To estimate activity, assay medium (RPM1640 supplemented with 5% FBS) was prepared, and M-07e cells were harvested by centrifugation, then washed with RPM1640 three times. The cells were resuspended in the assay medium, adjusted to 8×10^4 cells/ml in T-75 flask, and cultured for 24 hours in an atmosphere of 5% CO_2 . Again, the cells were harvested and adjusted to 1×10^5 cells/ml. 100 μl aliquots of the cell suspension were added to 96-well plates. Eight-step concentrations (100.0 ~ 0.78125 ng/ml) of standard material (rhTPO, 25 μg) were prepared by serial dilution with RPMI1640 medium, and CHO cell-derived native hTPO was employed as control. Total 11 species of hTPO derivatives (from 40429 to 40439) were prepared at the concentration of 1.5625, 6.25 and 25 ng/ml. A 100- μl aliquot of each sample per well was added, adjusting to 200 μl /well. After incubated for 20 hours in an atmosphere of 5% CO_2 , the cells were fed with 1 μCi (37 kBq) of ^3H -Thymidine and further incubated for 4 hours. Then, cells were harvested using cell harvester equipped with a glass fiber filter, which was washed with PBS seven times.

The filters in which cells were harvested were put in counting vials one by one, and ^3H -radioactivities emitted from each sample were measured with a liquid scintillation counter. Riasmart software was used to calculate the half-maximal concentration of standards, control and samples.

All derivatives showed similar patterns of activities stimulating M-07e cell proliferation. At the concentration of 25 ng/ml, 8 species of derivatives 40429, 40430, 40432, 40433, 40434, 40437, 40438 and 5 40439 showed similar or higher activities than native hTPO did, their activities amounting to 117, 135, 120, 131, 97, 121, 166 and 133% of native hTPO activity (see FIG. 4).

10 Example 5: In vivo activities of hTPO derivatives
isolated from CHO cells

In vivo hTPO assay was carried out where platelet levels were determined in the mice treated with various hTPO derivatives of this invention, and FIG. 6, 7a and 15 7b give the results. 7-week female Balb/c mice (Charles River, Japan) were adapted in a conditioning room ($24 \pm 1^{\circ}\text{C}$, 55% R.H., lighting for 12 hours, from 7:00 a.m. to 7:00 p.m.) for a week. The 8-week mice were brought to the assay and kept in the domestication 20 room during the test.

The mice were randomly divided into groups comprising 5 mice on the basis of weights. The groups were specified as groups treated with medium only, treated with native hTPO, treated with each hTPO 25 derivative of this invention, or not treated,

respectively.

Various hTPO derivatives (36 µg/kg or 10 µg/kg) were subcutaneously administered to the mice in single injection, and the blood samples of mice were collected everyday from day 0 (the day of injection) to day 10. Samples were collected from abdominal vena cava within 24 hours after administration. Whole blood in EDTA-treated tube was set on automatic hemocytometer (Cell dyn 3500, Abbott), by which platelet levels in samples were measured. The results were presented in 'mean ± standard error'.

On day 3, native hTPO stimulated an increase in platelet level. The platelet level reached a maximum on day 5 and came to normal level on day 10. All derivatives were found to stimulate an increase in platelet level, and derivatives 40433, 40434, 40449 and 40458 produced equal or higher platelet levels than native hTPO did. Especially, 40433 showed approximately 34% higher maximal in vivo activity of platelet production on day 5 than native hTPO, and 80% or more in total.

Comparative Example 1: in vivo activity of native hTPO

FIG. 5 shows the platelet level in a mouse that was treated with native hTPO derived from animal cells. 7-week female Balb/c mice (Charles River, Japan) were

adapted in a conditioning room ($24 \pm 1^{\circ}\text{C}$, 55% R.H., lighting for 12 hours, from 7:00 a.m. to 7:00 p.m.) for a week. The 8-week mice were brought to the assay and kept in the domestication room during the test.

5 The mice were randomly divided into groups comprising 5 mice on the basis of weights. The groups were specified as groups treated with medium only, treated with native hTPO, or not treated, respectively. Various concentrations (1, 5 and 10 $\mu\text{g/kg}$) of native
10 hTPO were subcutaneously administered in single injection, and the blood samples of mice were harvested on day 4, 8 and 10 (where day 1 is the day of injection). Sample was harvested from abdominal vena cava within 24 hours after administration. Whole blood
15 in EDTA-treated tube was set on automatic hemocytometer (Cell dyn 3500, Abbott), by which platelet levels in samples were measured. The results were presented in 'mean \pm standard error'. Native hTPO stimulated an increase in platelet level from day 4. The platelet
20 level reached a maximum on day 8 and came down to 80% of the maximal value on day 10.

Example 6: Construction of dhfr expression vectors
containing hTPO derivative cDNAs, and selection of
mammalian cell lines expressing them

(6-1) Construction of dhfr expression vectors
5 containing hTPO derivative cDNAs

According to the result of Example 5, dhfr
expression vectors were constructed, which
corresponding to the derivatives 40433, 40434, 40449
and 40458.

10 At first, *Bam*HI linker was inserted into pSV-dhfr
(ATCC 37146) containing dhfr gene. To prepare *Bam*HI
linker, two oligonucleotides (SEQ ID NO: 27 and NO: 28)
were phosphorylated and then annealed to hybridize with
each other. Particularly, T4 polynucleotide kinase
15 (NEB, Cat. No. 201S) was used in the phosphorylation
reaction at 37°C for 3 hours. In the annealing reaction,
the equimolar oligonucleotides were mixed and placed at
94°C for 2 min, then the mixture was stepwisely cooled
down from 65°C to 37°C with the temperature decreased
20 by 0.2°C per 30 sec. The vector pSV2-dhfr was
restricted with *Pvu*II and *Sph*I enzymes, then the *Bam*HI
linker was connected with the fragment of pSV2-dhfr.
The resulting vector was digested with *Bam*HI enzyme in
order to prepare the 1710-bp fragment containing dhfr
25 gene.

After the expression vector pCDT containing wild-type hTPO gene was digested with *Bgl*II enzyme, the 1710-bp fragment were inserted into the pCDT. The resulting dhfr expression vector expressing native hTPO was designated pDCT (see FIG. 8).

To dhfr expression vectors corresponding to 5 derivatives, two oligonucleotides (SEQ ID NO: 29 and NO: 2) were employed as PCR primers. Except for primers, the PCR was performed under the same condition as in Example 1. Amplified DNA sequences encoding hTPO derivatives were cut with *Kpn*I and *Eco*RI enzymes, and then inserted into the *Kpn*I-*Eco*RI site of the pDCT vector. The resulting vectors were designated pD40433, pD40434, pD40449 and pD40458, respectively.

15

(6-2) Transfection into CHO/dhfr(-) cell line and gene amplification

The dhfr expression vectors of Example 6-1 were transfected into animal cell line CHO/dhfr(-) (ATCC CRL-9096) according to the transfection procedure of Example 2. IMDM medium (Gibco-BRL, Cat. No. 12200-036) was used for the transfection, and IMDM medium supplemented with 10% dialyzed FBS (Gibco-BRL, Cat. No. 26300-061) for subsequent culture.

25

To select transformed line, the cells were added to 96-well microtiter plates (1×10^5 cells/well) in 48 hours after transfection, and cultured for 10-14 days

in medium containing 500 µg/ml zeocin. Zeocin-resistant colonies were isolated, and the 10-20 cell lines producing higher expression levels were selected by ELISA quantification.

5 The selected cell lines were subcultured in medium containing 20 nM MTX (Methotrexate, Sigma, Cat. No. M8407) to amplify hTPO gene. In detail, the cells were cultured in T-25 flask until flask was saturated with the cells. One-fifth of the saturated cells were
10 subcultured, then 1/10 and 1/15, successively. Amplification finished when T-25 flask was saturated with cells in 3-4 days after the 1/15 subculture. Cell lines producing highest expression levels were selected by ELISA from amplified cell lines in 20 nM MTX. The
15 cell lines were used to prepare samples for in vivo hTPO assay.

Example 7: Expression of native hTPO and derivatives thereof in CHO/dhfr(-) cells, and their purification

20 To prepare native hTPO and derivatives thereof, the cell lines of Example 6 were cultured in Cell Factory (Nunc, Cat. No. 170069) on 4-liter scale. Each cell line (5×10^4 cells/ml) was transferred into Cell Factory containing IMDM medium supplemented with 10%
25 FBS. Cultured for 72 hours, the cells were washed once

with PBS and then cultured in DMEM/Ham F-12 medium. After the cells were further cultured at 37°C for 96 hours in an atmosphere of 5% CO₂, supernatants obtained from the culture were brought to purification steps.

5 After XK26/20 column (Amersham-pharmacia, Cat. No. 18-1000-72) was filled with 50 ml of CM Affi-Gel blue resin (Bio-Rad, Cat. No. 153-7304), the column was washed with buffer A (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) overnight. 4-liter of the
10 culture supernatants was loaded and passed through the column with the flow rate of 5 ml/min, and was monitored by spectrophotometry at UV wavelength 280nm. After the whole culture supernatant was distributed throughout the column, the column was washed with
15 buffer B (10 mM sodium phosphate, 2 M urea, pH 7.4) until the UV absorption dropped to basal level. Bound proteins including hTPO were eluted with buffer C (10 mM sodium phosphate, 2 M urea, 1 M sodium chloride, pH 7.4), and this fraction was applied to subsequent
20 phenylsepharose column chromatography. XK26/20 column was filled with 50 ml of phenylsepharose CL4B resin (Sigma, Cat. No. P7892) and then washed with buffer C overnight. The fraction eluted from CM Affi-Gel blue column was applied to the phenylsepharose column with
25 flow rate of 3 ml/min and monitored by spectrophotometry at UV wavelength 280 nm. After the whole culture supernatant was distributed throughout

the column, the column was washed with buffer C until the UV absorption dropped to basal level. Proteins bound to resin were eluted with buffer B and this fraction was applied to subsequent hydroxylapatite column chromatography. XK16/20 column (Amersham-pharmacia, Cat. No. 18-8773-01) was filled with 10 ml of hydroxylapatite resin (Bio-Rad, Cat. No. 130-0420) and washed with buffer D (10 mM sodium phosphate, 2 M urea, pH 6.8) overnight. The fraction eluted from the phenylsepharose column was adjusted to pH 6.8 with 5 N HCl and then applied to hydroxylapatite column with flow rate of 3 ml/min. Since hTPO is not bound to hydroxylapatite resin, the unbound fraction was reserved. The column was washed with buffer D until the UV absorption dropped to basal level. Then, impure proteins bound to resin were eluted with buffer E (0.5 M sodium phosphate, 2 M urea, pH 6.8). The obtained hTPO fraction was concentrated to 10-ml volume using Econo-Pac Q cartridge (Bio-Rad, Cat. No. 732-0021), and then dialyzed in 10 mM sodium phosphate for 24 hours to eliminate salts and urea. Each fraction in the purification steps was visualized through SDS-PAGE and silver staining (see FIG. 9), where Silver-stain Plus kit (Bio-Rad, Cat. No. 161-0449) was used in accordance with the manufacturer instruction.

In vivo hTPO assay was performed with the purified hTPO derivatives (dose: 10 µg/kg) according to the

method of Example 5. All derivatives were found not only to stimulate an increase in platelet level, but also to produce higher platelet levels than native hTPO did. Particularly, 40433, 40434, 40449, and 40458 showed 77%, 91%, 26%, and 79% higher activities for total 10 days after administration than native hTPO, respectively (see FIG. 10).

Example 8: Characterization of hTPO derivatives:
verifying the introduction of sugar chains and
examining the stability of hTPO derivatives

To investigate whether additional sugar chains were introduced into the hTPO derivatives, SDS-PAGE and Western blot analysis was performed. If sugar chains are introduced, the molecular weights of hTPO derivatives will be heavier than that of native hTPO.

Purified native hTPO and derivatives thereof were loaded into wells in 10 20% gradient tricine polyacrylamide gel (Novex, Cat. No. EC66252), which was run at a voltage of 10 V/cm. After electrophoresis, the proteins fractionated on the gel were transferred onto a nitrocellulose filter. The filter was incubated for 1 hour in TBS (pH 7.5) containing 5% non-fat dried milk, and then further incubated for 18 hours with goat anti-hTPO polyclonal antibody (R&D system, Cat. No. AB-

288-NA) diluted in TBS (1:1000). The filter was subsequently incubated for 2 hours with a secondary antibody, alkaline phosphatase-conjugated anti-goat IgG (Sigma, Cat. No. A4187) diluted in TBS (1:10000). The coloring substrate BCIP/NBT (Sigma, Cat. No. B5655) was used for detecting hTPO band. In result, molecular weights of purified hTPO derivatives were heavier than that of native hTPO, depending on the number of sugar chains introduced (FIG. 11).

To evaluate the stability of hTPO derivatives, native hTPO and a hTPO derivative 40433 were digested with Thrombin, and then the time-dependent digestion patterns were observed. The hTPO derivative (50 µg/ml) was treated with Thrombin (5 units/ml, Sigma, Cat. No. T6759) at 37°C for 0.5, 1, 2, 3, 4, or 6 hours. Then, SDS-PAGE and Western blot analysis was performed to observe the digestion patterns. Native hTPO was strikingly degraded in 30 min after treatment with Thrombin, while the derivative 40433 was digested in 4 hours (see FIG. 12). This result verified that the derivative 40433 is more stable than native hTPO, which can be explained from the sugar chain introduced.

INDUSTRIAL APPLICABILITY

As shown above, the hTPO derivatives of this invention induce the production of platelet precursor

cells in vivo, and thus are useful for the treatment of thrombocytopenia associated with anticancer therapies or bone marrow graft. Especially, the hTPO derivatives 40433, 40434, 40449 and 40458 show significantly higher efficacy inducing platelet production than native hTPO, providing various advantages. Since low dose of hTPO derivatives shows similar efficacy to native hTPO, small dose of hTPO can be infrequently administered to the patients suffering from thrombocytopenia. Therefore, use of derviatives of this invention will reduce the cost of treating the disease and will elevate the welfare of patients as well as the safety of the drug, with the inclusion of impure proteins excluded, owing to the small dose used.

15

Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.

25

PCT

9FPO-04-08

Original (for SUBMISSION) - printed on 30.06.1999 12:00:19 PM

0-1	Form - PCT/RO/134 (EASY) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)	
0-1-1	Prepared using	PCT-EASY Version 2.84 (updated 01.06.1999)
0-2	International Application No	PCT/KR 99/ 00 3 47
0-3	Applicant's or agent's file reference	9FPO-04-08
1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
1-1	page	19, 48
1-2	line	4, 25
1-3	Identification of Deposit	
1-3-1	Name of depositary institution	Korean Collection for Type Cultures
1-3-2	Address of depositary institution	52, Oun-dong, Yusong-Ku, Taejon 305-333, Republic of Korea
1-3-3	Date of deposit	17 June 1998 (17.06.1998)
1-3-4	Accession Number	KCTC 0495BP
1-4	Additional Indications	NONE
1-5	Designated States for Which Indications are Made	all designated States
1-6	Separate Furnishing of Indications These indications will be submitted to the International Bureau later	NONE
2	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
2-1	page	22, 49
2-2	line	21, 2
2-3	Identification of Deposit	
2-3-1	Name of depositary institution	Korean Collection for Type Cultures
2-3-2	Address of depositary institution	52, Oun-dong, Yusong-Ku, Taejon 305-333, Republic of Korea
2-3-3	Date of deposit	08 June 1999 (08.06.1999)
2-3-4	Accession Number	KCTC 0630BP
2-4	Additional Indications	NONE
2-5	Designated States for Which Indications are Made	all designated States
2-6	Separate Furnishing of Indications These indications will be submitted to the International Bureau later	NONE
3	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
3-1	page	22, 49
3-2	line	21, 6

PCT

9FPO-04-08

Original (for SUBMISSION) - printed on 30.06.1999 12:00:19 PM

3-3	Identification of Deposit	
3-3-1	Name of depositary institution	Korean Collection for Type Cultures
3-3-2	Address of depositary institution	52, Oun-dong, Yusong-Ku, Taejon 305-333, Republic of Korea
3-3-3	Date of deposit	08 June 1999 (08.06.1999)
3-3-4	Accession Number	KCTC 0631BP
3-4	Additional Indications	NONE
3-5	Designated States for Which Indications are Made	all designated States
3-6	Separate Furnishing of Indications	NONE
	These indications will be submitted to the International Bureau later	
4	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
4-1	page	22, 49
4-2	line	22, 10
4-3	Identification of Deposit	
4-3-1	Name of depositary institution	Korean Collection for Type Cultures
4-3-2	Address of depositary institution	52, Oun-dong, Yusong-Ku, Taejon 305-333, Republic of Korea
4-3-3	Date of deposit	08 June 1999 (08.06.1999)
4-3-4	Accession Number	KCTC 0632BP
4-4	Additional Indications	NONE
4-5	Designated States for Which Indications are Made	all designated States
4-6	Separate Furnishing of Indications	NONE
	These indications will be submitted to the International Bureau later	

FOR RECEIVING OFFICE USE ONLY

0-4	This form was received with the international application: (yes or no)	YES
0-4-1	Authorized officer	Lee, Seung Jong <i>[Signature]</i>

FOR INTERNATIONAL BUREAU USE ONLY

0-5	This form was received by the international Bureau on:	
0-5-1	Authorized officer	

What is claimed is

1. Human thrombopoietin derivative which derived from human thrombopoietin (hTPO) described by SEQ ID NO: 30; which has at least one additional N-linked glycosylation site; and which is selected from the group comprising:
 - [Asn¹⁰⁸] hTPO;
 - [Asn¹¹⁷] hTPO;
 - 10 [Asn¹⁴⁷] hTPO;
 - [Asn¹⁵³] hTPO;
 - [Asn¹⁶⁴] hTPO;
 - [Asn¹⁶³] hTPO;
 - [Asn¹¹⁷, Asn¹⁴⁷] hTPO;
 - 15 [Asn¹¹⁷, Asn¹⁶⁴] hTPO;
 - [Asn¹⁰⁸, Asn¹⁴⁷] hTPO;
 - [Asn¹⁰⁸, Asn¹⁶⁴] hTPO;
 - [Asn¹⁴⁷, Asn¹⁶⁴] hTPO;
 - [Asn¹¹⁷, Asn¹⁴⁷, Asn¹⁶⁴] hTPO;
 - 20 [Asn¹⁰⁸, Asn¹⁴⁷, Asn¹⁶⁴] hTPO;
 - [Asn¹⁰⁸, Asn¹¹⁷, Asn¹⁶⁴] hTPO;
 - [Asn¹⁵³, Asn¹⁶⁴] hTPO;
 - [Asn¹⁶³, Ser¹⁶⁴] hTPO;
 - [Asn¹⁶³, Thr¹⁶⁴] hTPO;
 - 25 [Asn¹⁵³, Ser¹⁶³, Asn¹⁶⁴] hTPO;
 - [Asn¹⁵³, Thr¹⁶³, Asn¹⁶⁴] hTPO;

[Asn¹⁵⁹, Ser¹⁶¹, Asn¹⁶⁴] hTPO;

[Asn¹⁵⁹, Thr¹⁶¹, Asn¹⁶⁴] hTPO;

[Asn¹⁶⁶, Ser¹⁶⁷] hTPO;

[Asn¹⁶⁶, Thr¹⁶⁷] hTPO; and

5 [Asn¹⁶⁴, Asn¹⁶⁷] hTPO.

2. The human thrombopoietin derivative of claim 1
which is [Asn¹⁶⁴] hTPO, [Asn¹⁶⁷] hTPO, [Asn¹⁶⁶, Asn¹⁶⁷,
Asn¹⁶⁴] hTPO, or [Asn¹⁶⁷, Asn¹⁶⁴] hTPO.

10

3. Recombinant gene encoding human thrombopoietin
derivative of claim 1.

4. Recombinant gene encoding human thrombopoietin
15 derivative of claim 2.

5. Eukaryotic expression vector containing the
recombinant gene of claim 3.

20 6. The eukaryotic expression vector of claim 5 which
is p40433, p40434, p40449, p40458, pD40433, pD40434,
pD40449, or pD40458.

7. Mammalian cell line CHO K-1/p40433 (Accession NO:
25 KCTC 0495BP) transfected with the expression vector
p40433 of claim 6.

8. Mammalian cell line CHO dhfr-/pD40434 (Accession
NO: KCTC 0630BP) transfected with the expression
vector pD40434 of claim 6.
- 5 9. Mammalian cell line CHO dhfr-/pD40449 (Accession
NO: KCTC 0631BP) transfected with the expression
vector p40449 of claim 6.
- 10 10. Mammalian cell line CHO dhfr-/pD40458 (Accession
NO: KCTC 0632BP) transfected with the expression
vector p40458 of claim 6.
- 15 11. Process of preparing the human thrombopoietin
derivative of claim 1 wherein a mammalian cell line
containing the recombinant gene of claim 3 is used
to obtain the human thrombopoietin derivative of
claim 1.
- 20 12. Pharmaceutical composition containing the human
thrombopoietin derivative of claim 1 which is used
for the treatment of thrombocytopenia.

FIG. 1

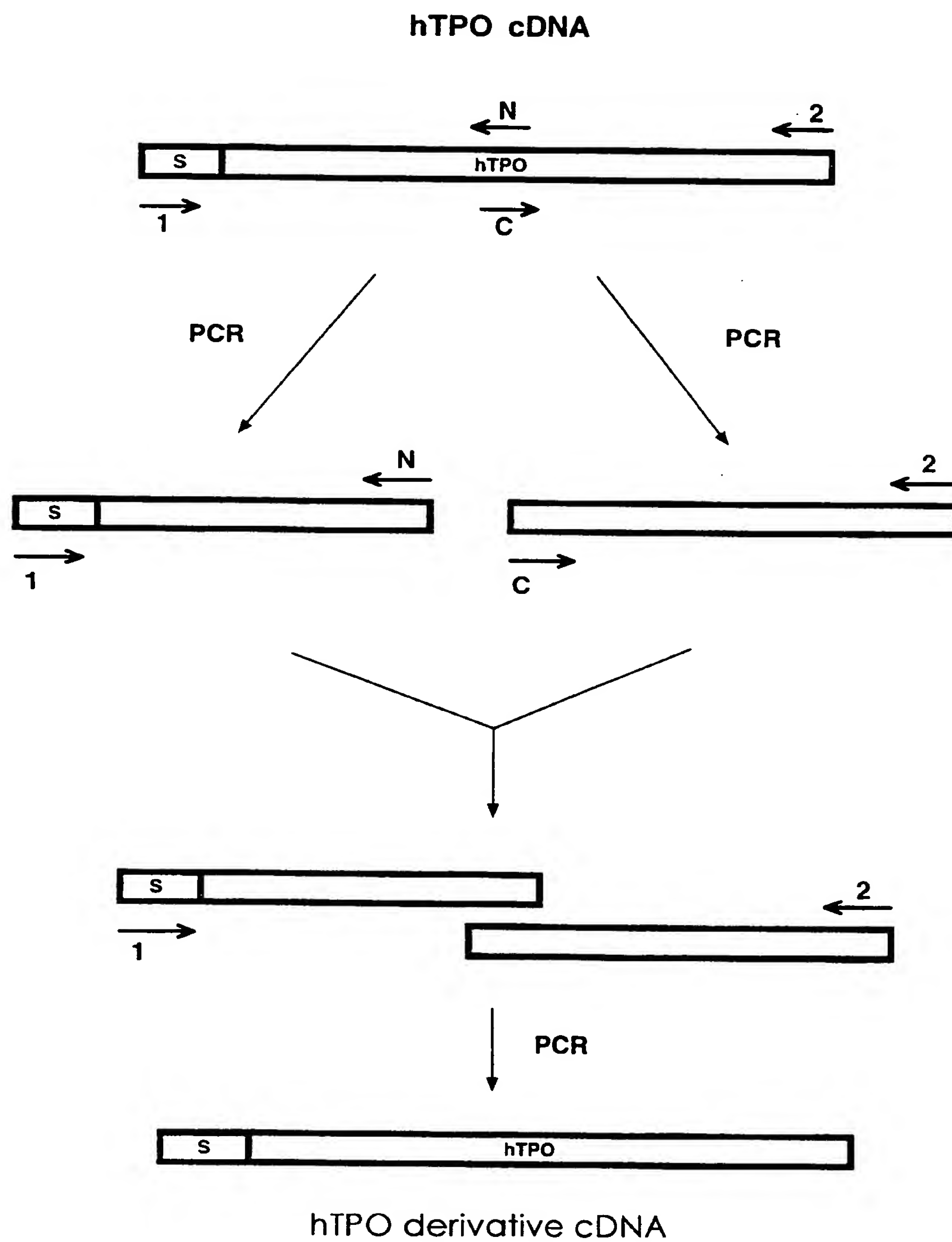


FIG. 2

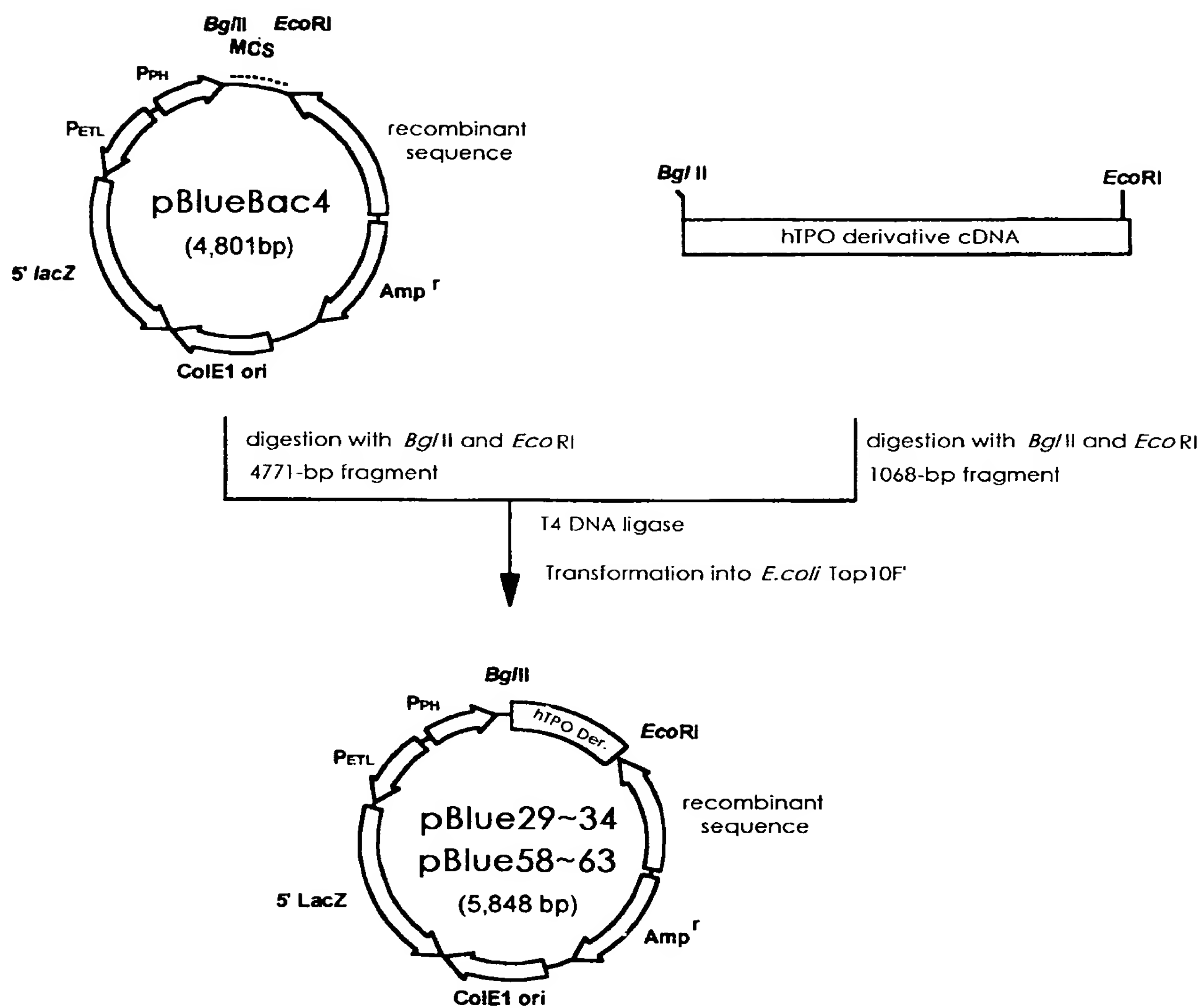


FIG. 3

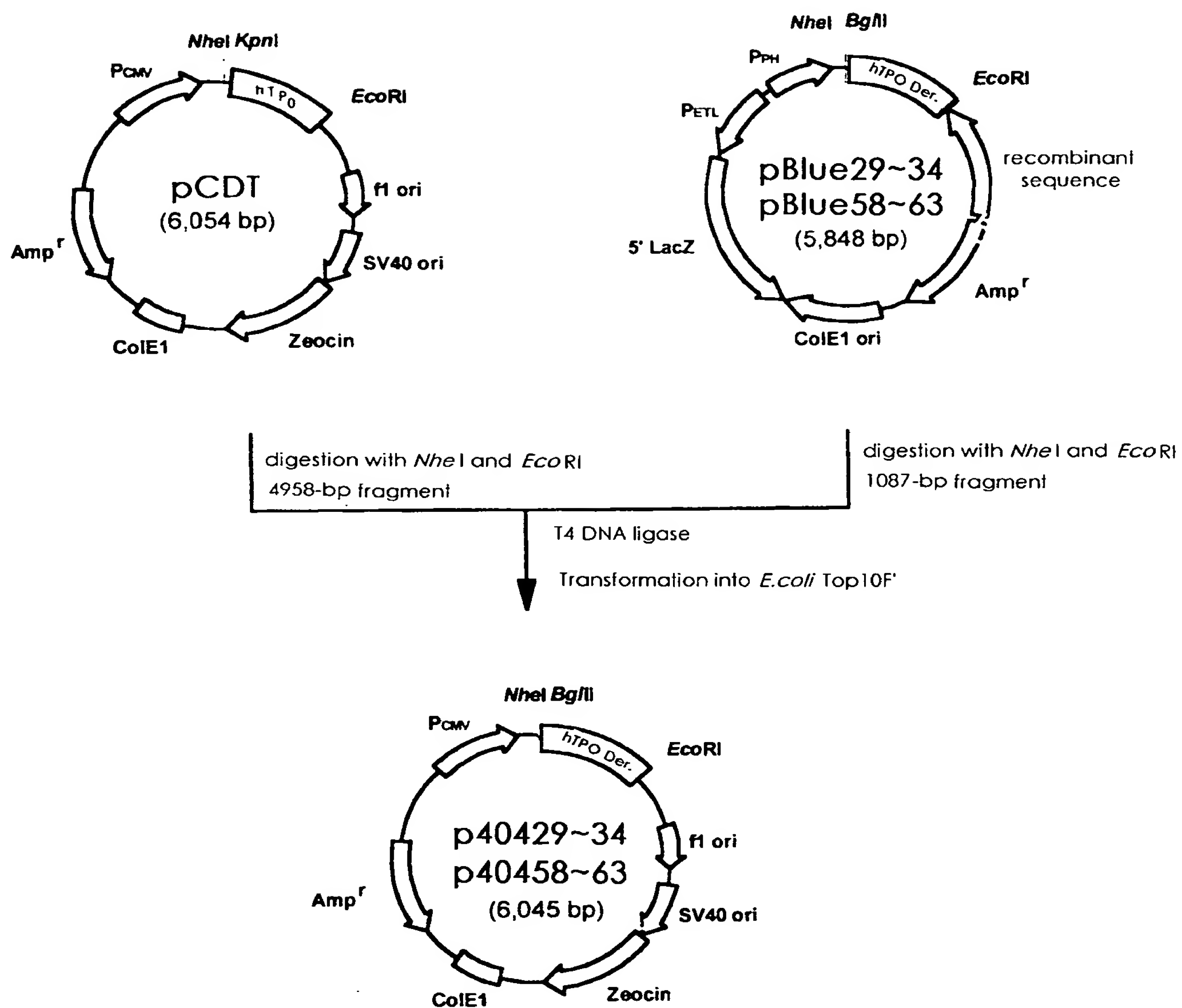


FIG. 4

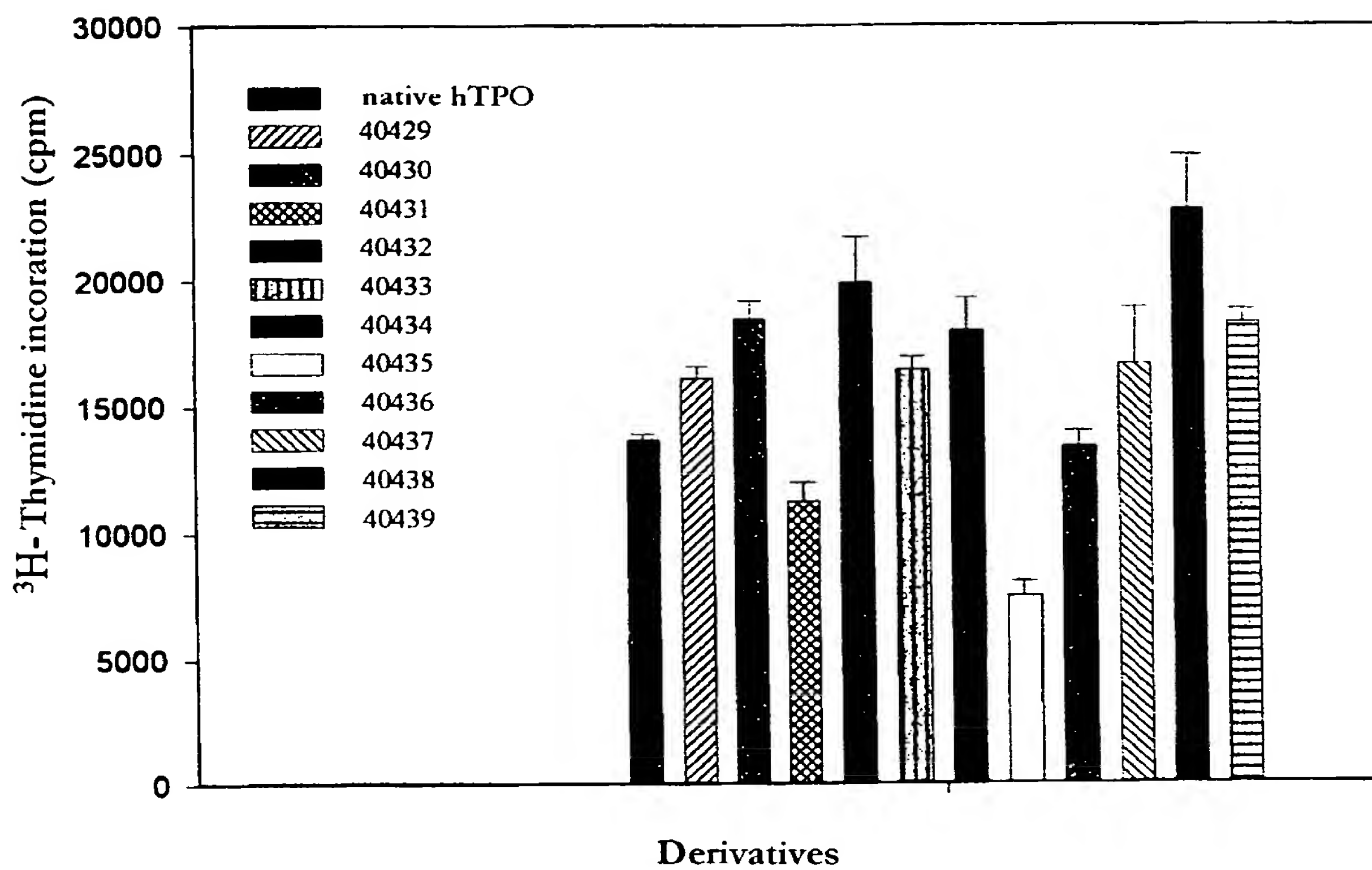


FIG. 5

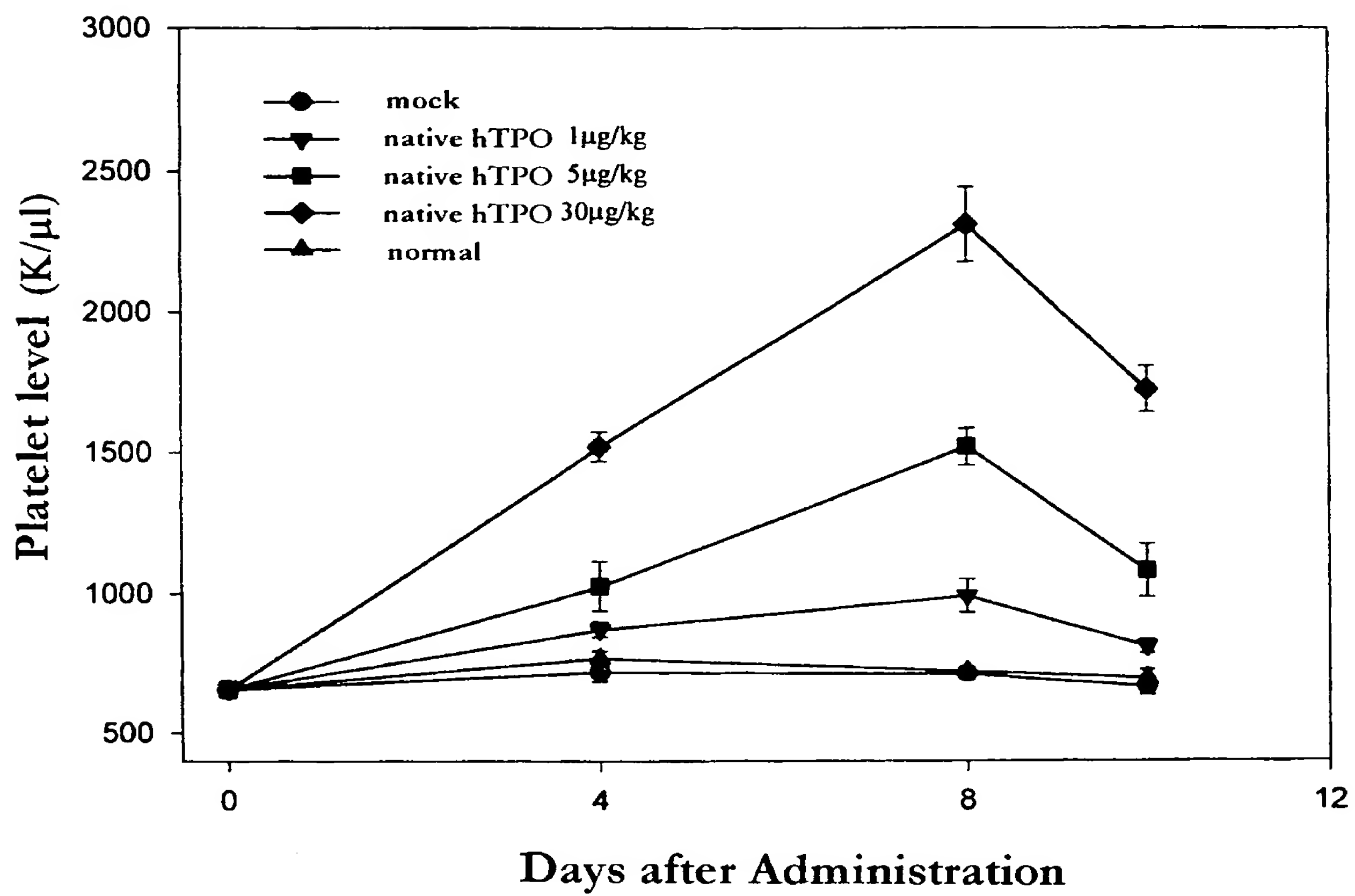


FIG. 6

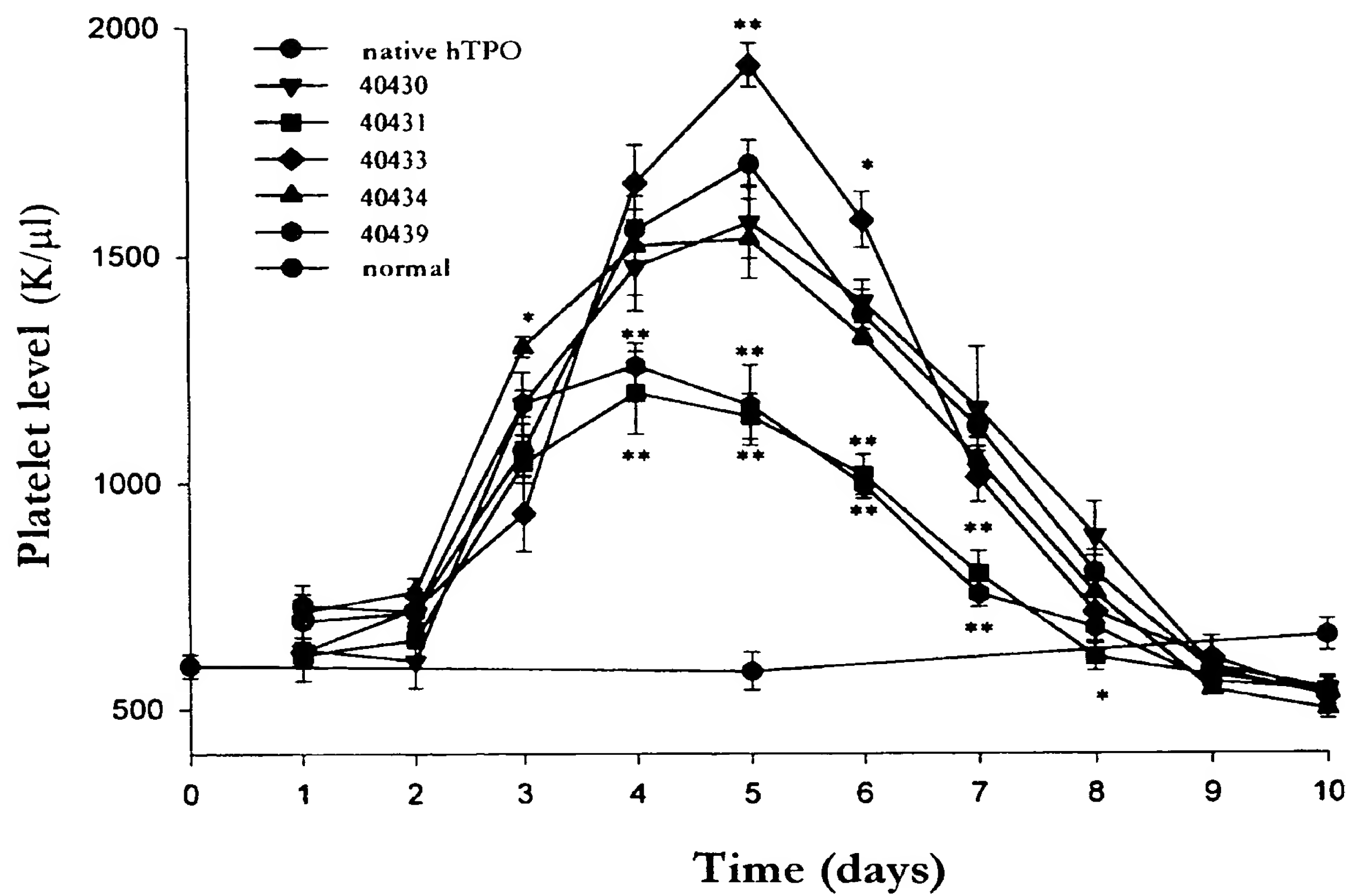


FIG. 7a

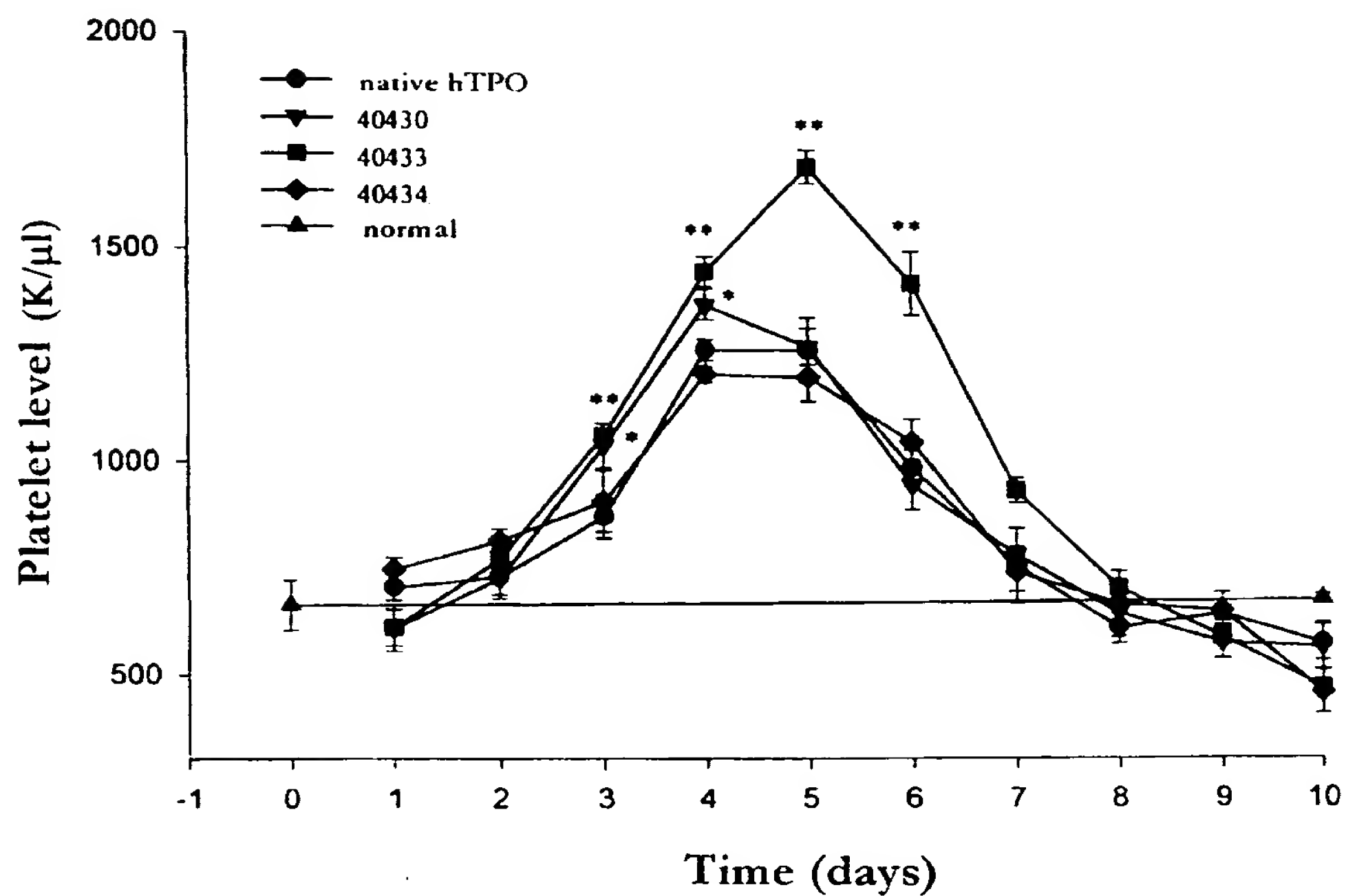


FIG. 7b

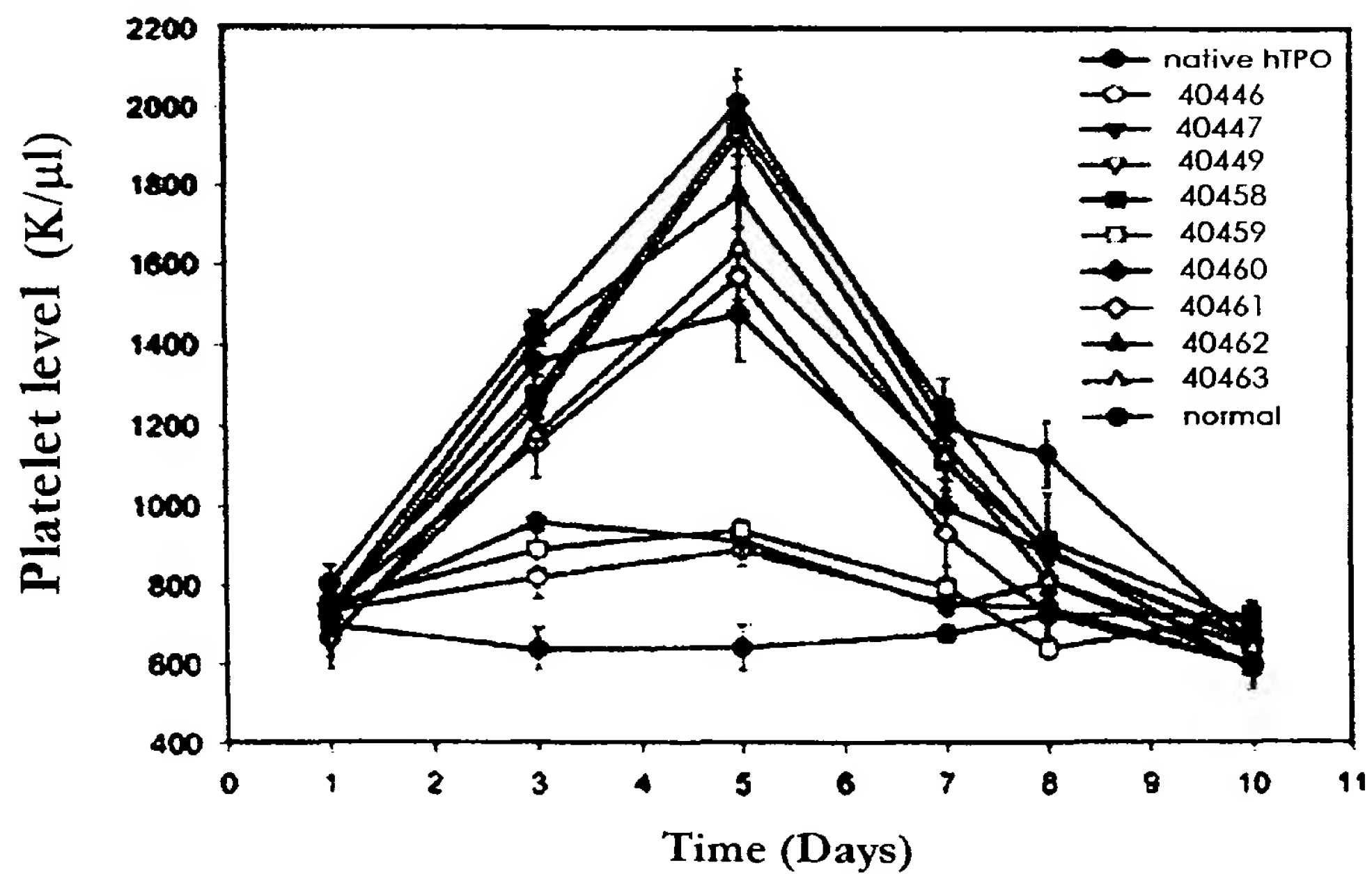


FIG. 8

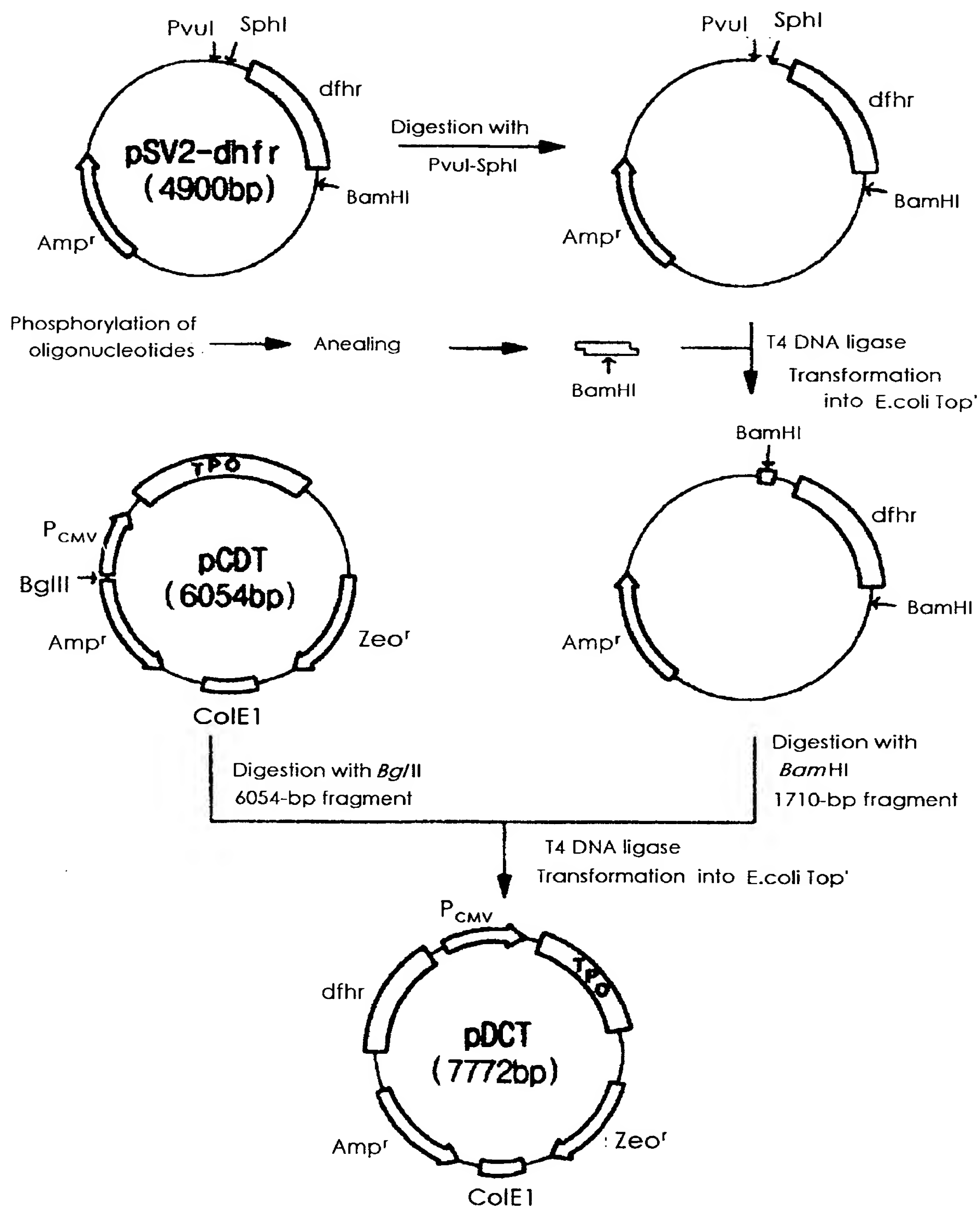


FIG. 9

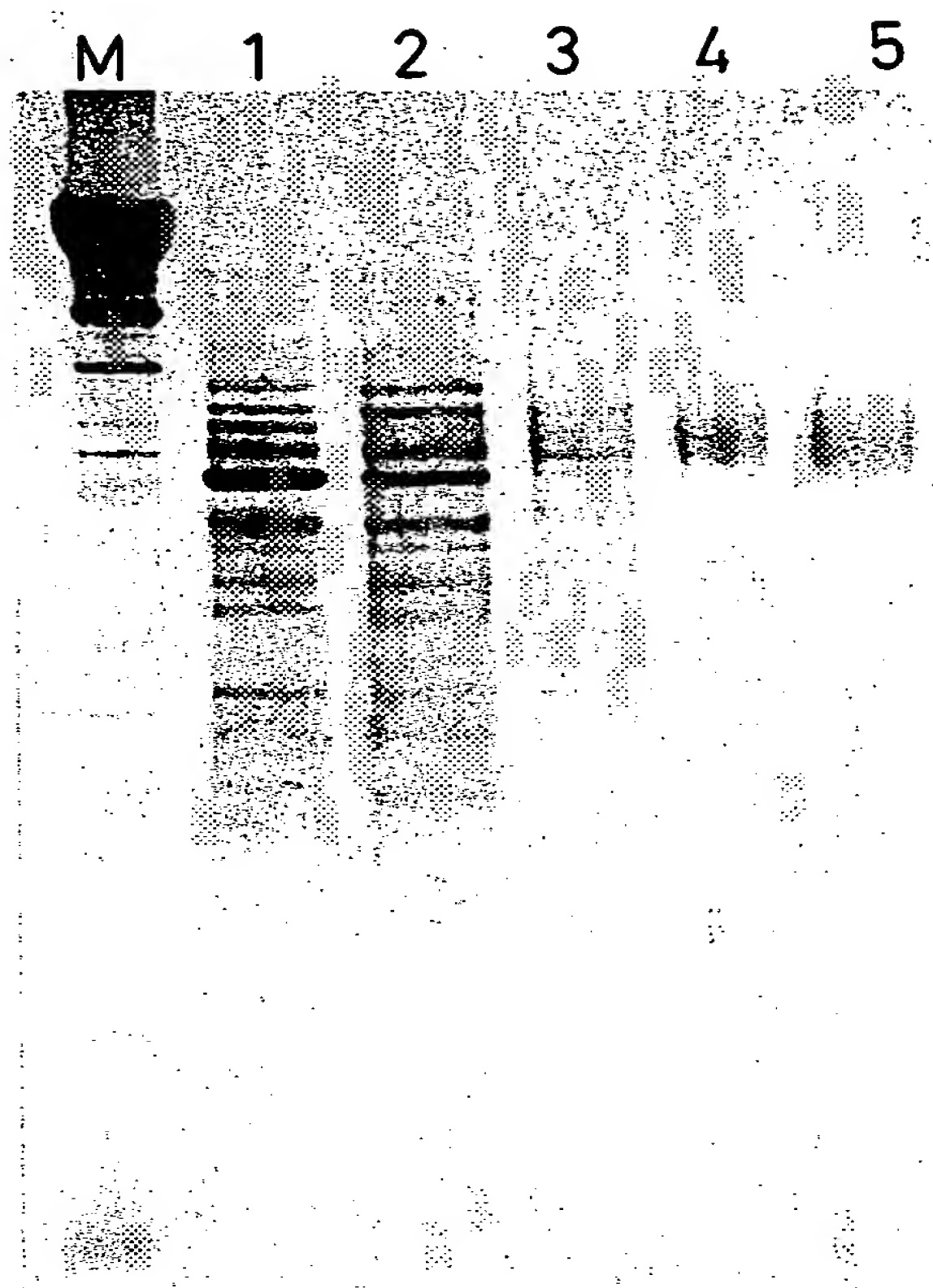


FIG. 10

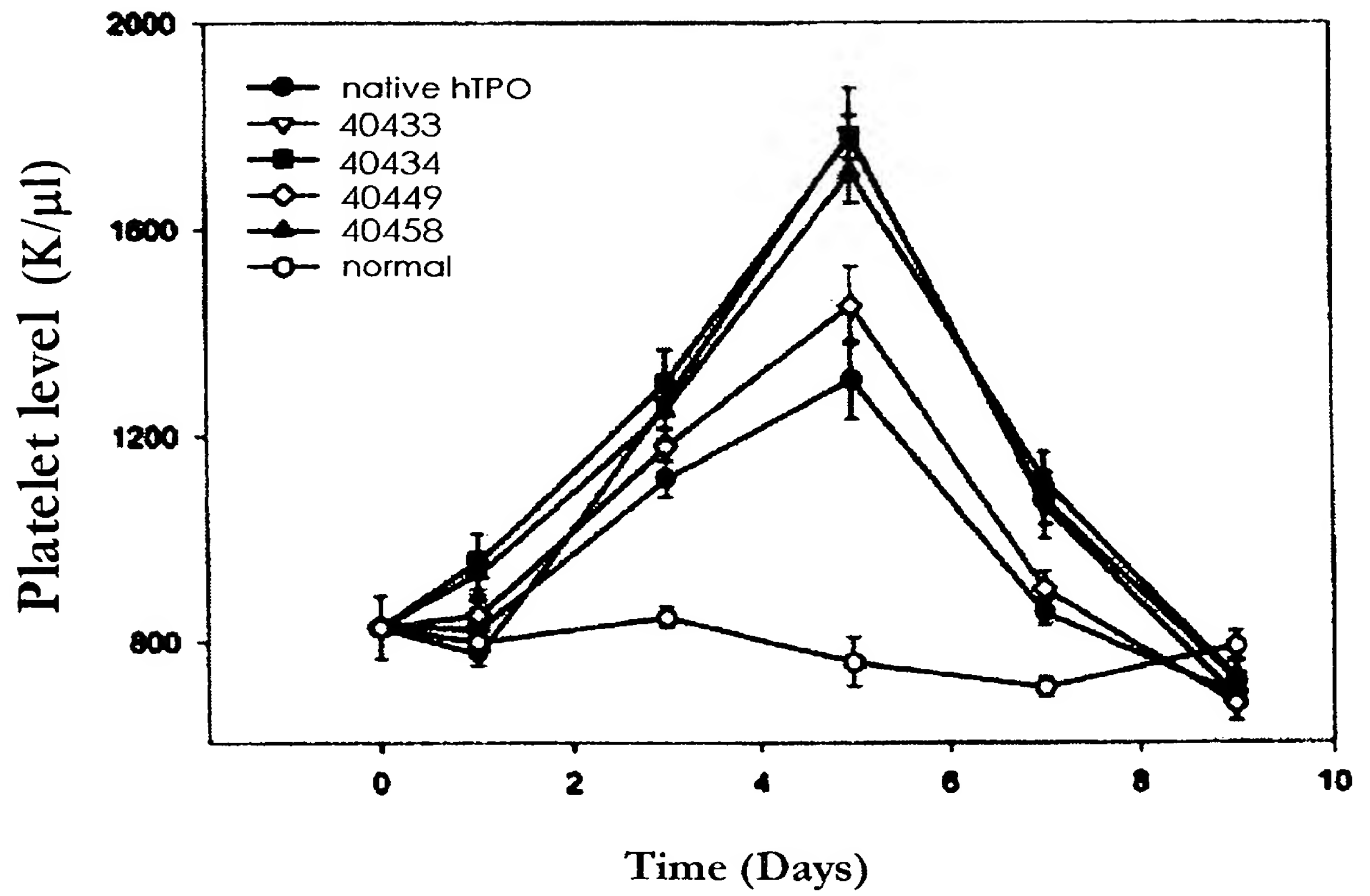


FIG. 11

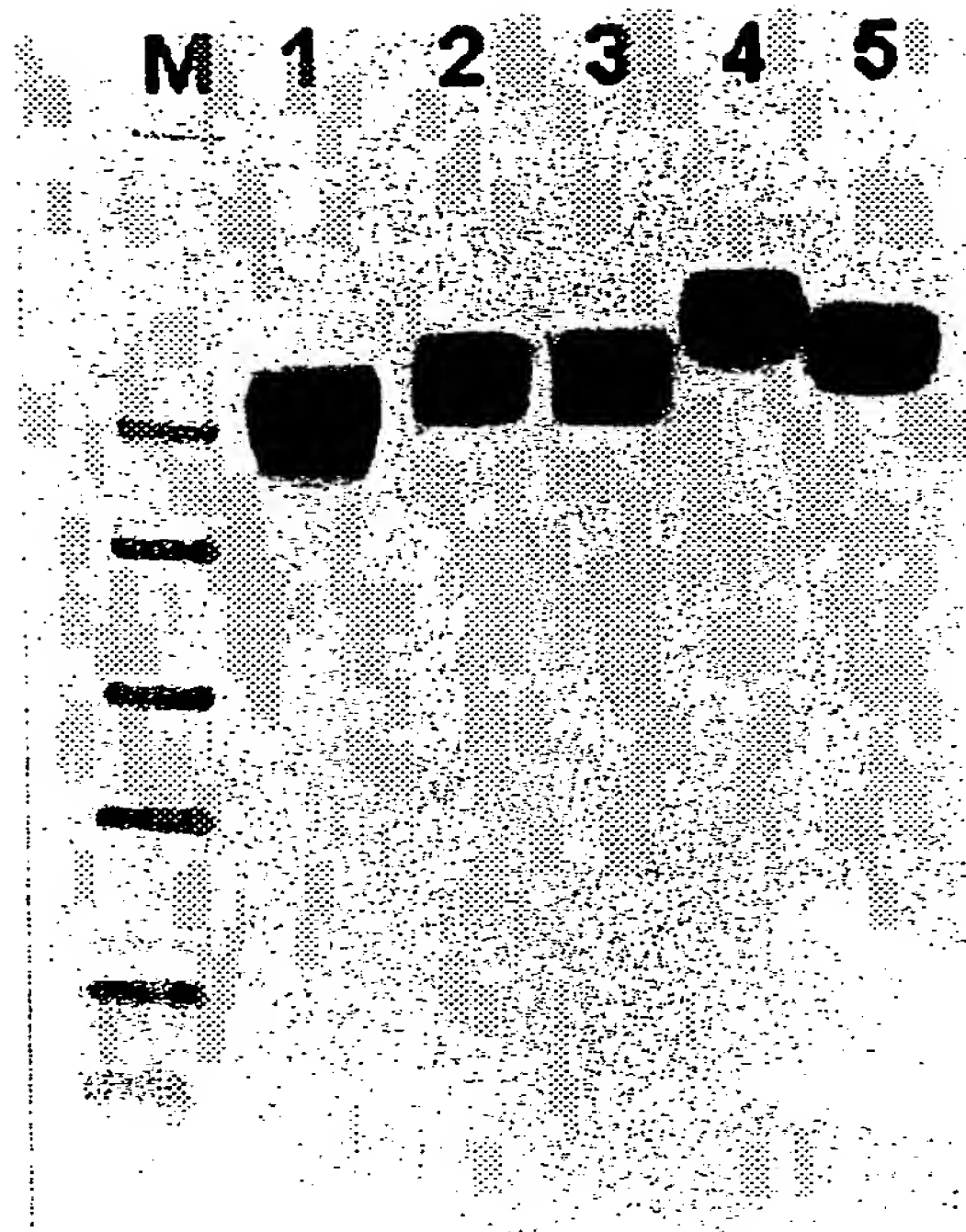


FIG. 12a

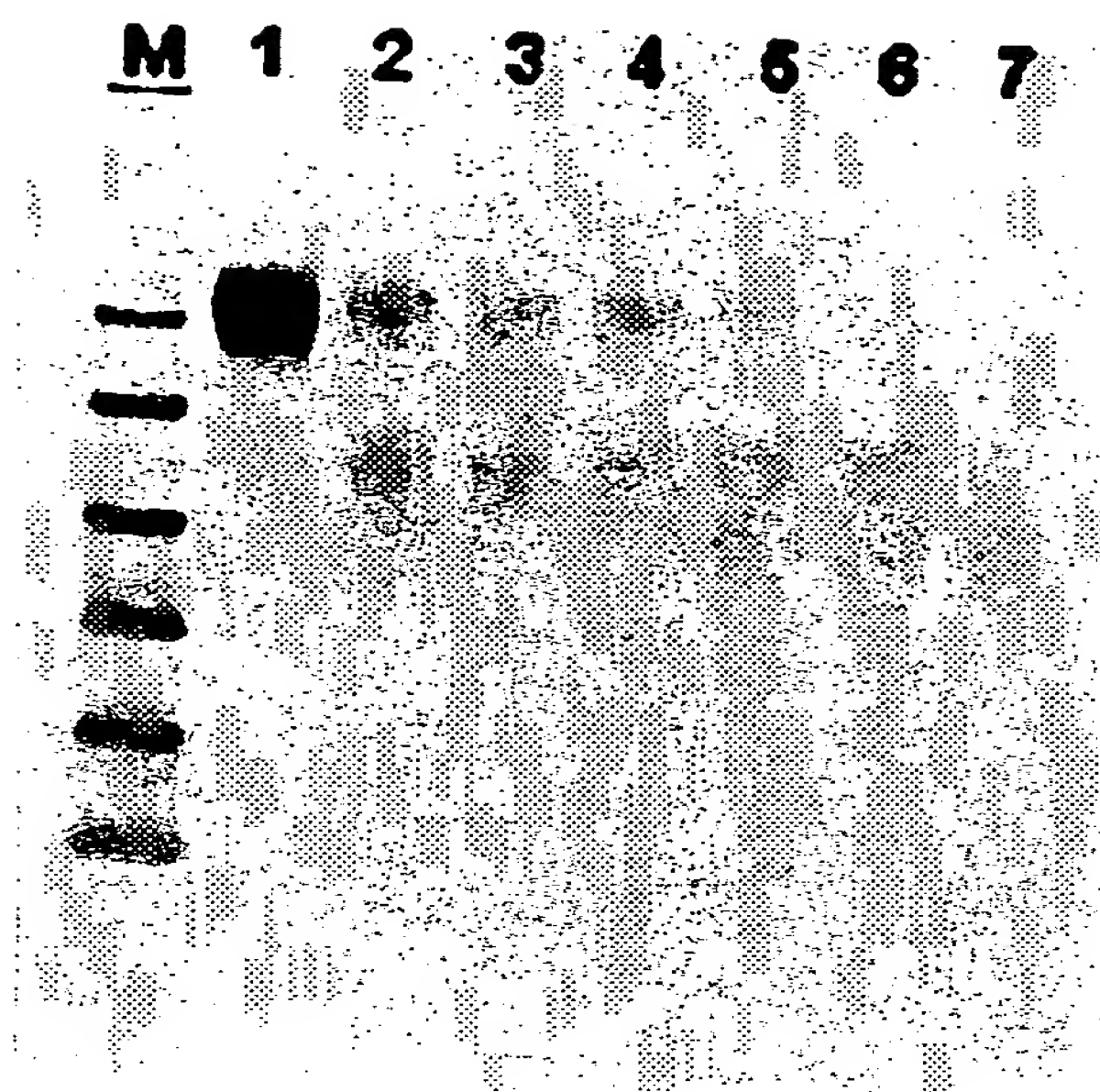
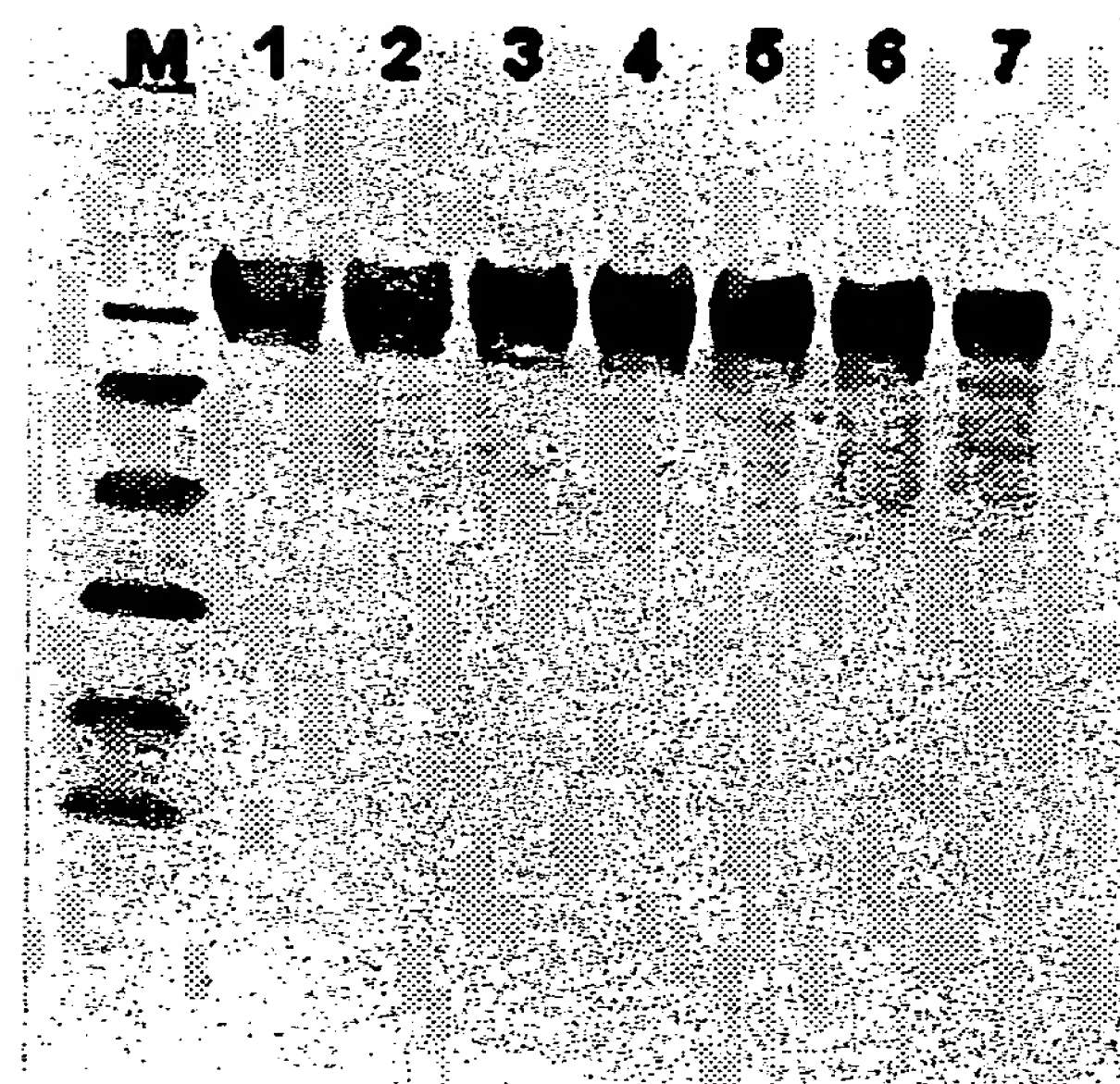


FIG. 12b



SEQUENCE LISTING

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<130> 9fpo-04-08

<150> KR 98-25935

<151> 1998-06-30

<150> KR 99-25143

<151> 1999-06-29

<160> 34

<170> KOPATIN 1.0

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<211> 23

<212> DNA

<213> Artificial Sequence

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<223> BglII-tagged primer corresponding to the N terminal sequence of hTPO protein

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<210> 2

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Gly Glu Trp Lys Thr Gln Met Glu Glu Thr Lys Ala Gln Asp Ile Leu
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Leu Gly Pro Thr Cys Leu Ser Ser Leu Leu Gly Gln Leu Ser Gly Gln
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Pro Pro Gln Gly Arg Thr Thr Ala His Lys Asp Pro Asn Ala Ile Phe
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 99/00347

A. CLASSIFICATION OF SUBJECT MATTER IPC ⁶ : C 12 N 15/19, 15/79, 5/10, A 61 K 38/19 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC ⁶ : C 12 N 15/19, 15/79, 5/10, A 61 K 38/19 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) WPI, EPODOC, CAS		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 25498 A2 (AMGEN INC.) 22 August 1996 (22.08.96), page 1; claims 1, 2, 4, 5, 7-21.	1 - 12
A	WO 96 17062 A1 (ZYMOGENETICS, INC.) 6 June 1996 (06.06.96), claims 1, 6-9, 19, 21, 23, 26, 28.	1, 3, 5, 7-12
A	WO 95 18858 A1 (GENENTECH, INC.) 13 July 1995 (13.07.95), pages 15-17, claims 1-4, 22, 24-26, 29-31, 36, 37, 40.	1, 3, 5, 11, 12
A	EP 0 668 352 A1 (KIRIN BREWERY COMPANY, LTD.) 23 August 1995 (23.08.95), claims 1, 17, 22, 30, 31, 33.	1, 3, 11, 12
A	Hoffman et al. Peptide, Disulfide, and Glycosylation Mapping of Recombinant Human Thrombopietin Ser 1 to Arg 246, Biochemistry 1996, 35 (47), 14849-14861 (Eng.). Columbus, Ohio, USA: Chemical abstracts, Vol. 126, No. 6, 10 February 1997, page 80, left column, the abstract no. 70279s.	1
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
* Special categories of cited documents: „A“ document defining the general state of the art which is not considered to be of particular relevance „E“ earlier application or patent but published on or after the international filing date „L“ document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) „O“ document referring to an oral disclosure, use, exhibition or other means „P“ document published prior to the international filing date but later than the priority date claimed „T“ later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention „X“ document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone „Y“ document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art „&“ document member of the same patent family		
Date of the actual completion of the international search 6 October 1999 (06.0.99)		Date of mailing of the international search report 05 November 1999 (05.11.99)
Name and mailing address of the ISA/AT Austrian Patent Office Kohlmarkt 8-10; A-1014 Vienna Facsimile No. 1/53424/200		Authorized officer Mosser Telephone No. 1/53424/437

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR 99/00347

Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche		Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
WD A2	9625498	22-08-1996	AU A1 49946/96 AU B2 691410 CA AA 2209298 CN A 1182453 EP A2 811064 JP T2 11500904 NZ A 303588 US A 5756083 WO A3 9625498 US A 5696250	04-09-1996 14-05-1998 22-08-1998 20-05-1998 10-12-1997 26-01-1999 25-02-1999 26-05-1998 20-03-1997 09-12-1997
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WD A1	9518858	13-07-1995	AU A1 15146/95 AU B2 704266 BE AF 1009021 BG A 100693 CA AA 2178482 CN A 1141061 CN A3 9601922 DE A1 19500030 DK A 1491/94 EP A1 738323 ES AA 2114786 ES BA 2114786 FI A0 962723 FI A 962723 FR A1 2714670 FR B1 2714670 GB A0 9425831 GB A1 2285446 GB B2 2285446 GR B 1002005 HR A1 941020 HU A0 9601805 HU A2 75657 HU A1 950002 IE B 72517 IL A0 112184 IL A0 950002 IL A1 950002 JP B1 1283770 JP T2 9508262 JP A2 10113186 LU A 88573 LV A 11632 LV B 11632 NL A 9500010 NO A0 962783 NO A 962783 NN A 278726 PT A 101627 PT B 101627 SG A1 47030 SG A 9420079 SK A3 875/96 US A 5830647 US A 9408487 US A 96118 US B 4120 ZA A 9410355	01-08-1995 15-04-1999 01-10-1995 30-01-1998 13-07-1995 22-01-1997 14-05-1997 06-07-1995 04-07-1995 23-10-1995 01-06-1998 01-01-1999 02-07-1996 03-09-1996 07-07-1997 10-01-1997 22-02-1995 12-07-1995 25-07-1995 27-10-1995 30-04-1997 28-08-1996 28-08-1997 12-07-1997 23-04-1997 15-03-1998 02-01-1998 03-07-1998 30-04-1998 26-08-1997 06-05-1999 01-06-1999 20-12-1998 20-04-1997 01-08-1995 02-07-1995 03-09-1995 26-06-1995 30-11-1995 29-11-1995 20-03-1998 28-02-1997 05-11-1997 03-11-1998 26-08-1997 27-12-1996 25-03-1997 09-07-1996

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR99/00347

EP A1 668352 23-08-1995

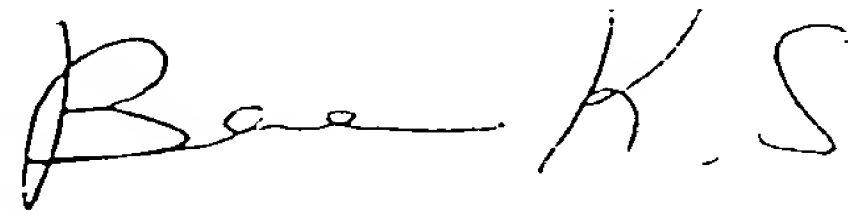
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WO	A2	9521919	17-08-1996
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CA	AA	2160391	17-08-1996
EP	A1	695355	07-02-1996
FI	A0	954689	13-10-1996
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RU	A1	411888	18-04-1996
RU	A0	112502	25-05-1996
JP	A2	8109195	30-04-1996
NO	A0	954058	12-10-1996
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ON	A	1131458	18-09-1996
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INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

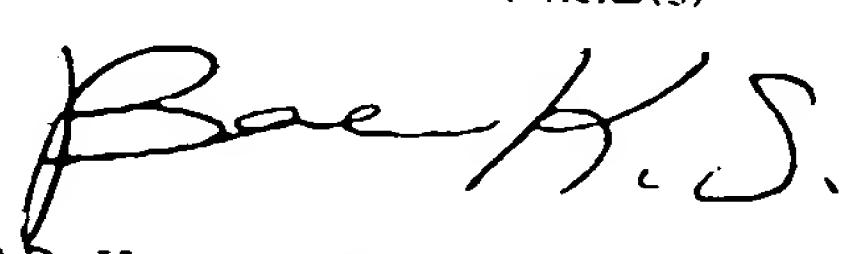
issued pursuant to Rule 7.1

TO: Yoon, Jae-Seung
Daewoong Pharmaceutical Co., Ltd. #163-3, Samsung-dong, Kangnam-ku,
Seoul 135-715, Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: CHO K-1/p40433	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCTC 0495BP
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: [x] a scientific description [] a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on Jun 17 1998 .	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on _____ and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on _____	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Korea Research Institute of Bioscience and Biotechnology Korean Collection for Type Cultures Address: KCTC, KRIBB #52, Oun-dong, Yusong-ku, Taejeon 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Kyung Sook Bae, Curator Date: Jun 22 1998

INTERNATIONAL FORM
RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1

TO: YOON, Jae-Seung
#163-3, Samsung-dong, Kangnam-ku, Seoul 135-090,
Republic of Korea

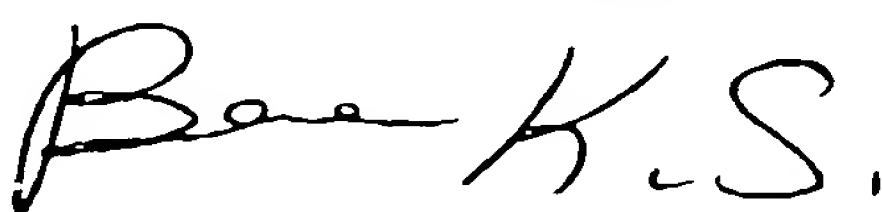
I. IDENTIFICATION OF THE MICROORGANISM	
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II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
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III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on Jun 08 1999 .	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on _____ and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on _____	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Korean Collection for Type Cultures Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s):  BAE, Kyung Sook, Director Date: Jun 21 1999

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO: YOON, Jae-Seung
#163-3, Samsung-dong, Kangnam-ku, Seoul 135-090,
Republic of Korea

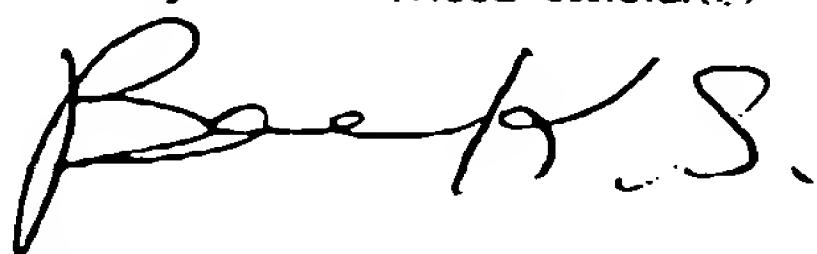
I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: CHO dhfr-/pD40449	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCTC 0631BP
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: [x] a scientific description [] a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on Jun 08 1999 .	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on _____ and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on _____	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Korean Collection for Type Cultures Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejeon 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s):  BAE, Kyung Sook, Director Date: Jun 21 1999

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO: YOON, Jae-Seung
#163-3, Samsung-dong, Kangnam-ku, Seoul 135-090,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: CHO dhfr-/pD40458	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCTC 0632BP
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: [x] a scientific description [] a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on Jun 08 1999 .	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on _____ and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on _____	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Korean Collection for Type Cultures Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s):  BAE, Kyung Sook, Director Date: Jun 21 1999